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CHANGES IN ELECTRICAL POTENTIAL IN NORMAL, CASTRATED, AND THEELIN-TREATED RATS¹

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The development of the Burr-Lane microvoltmeter (1) opened up new approaches to many fields of research. Although it has been known for some time (2) that electrical responses may be elicited from living systems, there have been few practical and stable instruments for accurately measuring minute electrical potentials. The question of the relationship of the reproductive system to bio-electric phenomena is of especial interest to endocrinologists. Burr, Hill, and Allen (3) have been able to detect the exact time of ovulation in the rabbit by the characteristic changes in the electrical potential. More recently, Burr and Musselman (4) working on electrical potential variations in women, have shown that there is a definite correlation between these daily potentials and the menstrual cycle. The experiments reported in this series of papers were carried out in an effort to determine the relationship between potentials of the normal and experimental oestrous cycle in the rat (5, 6, 7).

Sexually mature albino and hooded rats were used for the experiment.

While the potential determinations were being made, it was necessary to anesthetize the animal in order to keep it perfectly quiet. After trials with ether and various salts of barbituric acid, sodium amytal was found to be best for the purpose. The amytal² was prepared in a 3 per cent solution. This percentage was chosen because less than 1 cc. was enough for the largest animals in the colony and more than 0.2 cc. was required for the smallest. In using sodium amytal, where it is desirable to anesthetize a rat every day or more than once a day, the dose may be gradually

¹ Part of a dissertation presented for the degree of Doctor of Philosophy at Yale University.

² Kindly supplied by Doctor Peck of Eli Lilly & Co.

reduced. Rats may be kept under amytal for long periods of time. In some experiments several animals were kept under anesthesia for more than eight hours with apparently no ill effects. Small quantities (0.2 cc. of 3 per cent solution) must be injected about every two hours to prevent their returning to consciousness. During some of the experiments it was necessary to anesthetize an animal as many as four times in one day. This was done without any difficulty provided the amount of amytal given was considerably reduced after the first dose.

After a rat had been anesthetized, the hair was clipped from the region of the symphysis pubis. The animal was then placed on its back on a cork board and the region of the pubis moistened with salt solution. The salt bridge from one electrode was placed on the symphysis and the other inserted well into the vagina. Enough saline flowed into the vaginal cavity to insure contact with the wall.

In order to interpret the electrical potential readings, it was necessary to correlate each determination with the exact stage of the oestrous cycle. For this purpose, a vaginal smear was always made before each reading.

EXPERIMENTAL. The changes in electrical potential³ were studied in thirty-seven normal adult female rats through two to five consecutive oestrous cycles. Five hundred and nine readings, taken twenty-four hours apart, were made on these animals with normal oestrous cycles. Eighty-five cycles were followed, all but one of which were found to have what was considered a typical electrical potential curve (fig. 1).

Figure 1 shows a graph made from the twenty daily readings of a typical normal animal passing through four oestrous cycles of five days each. During late oestrus the vagina is negative to the symphysis pubis by many thousand microvolts. As the animal goes into post-oestrus, there is a sudden drop in the potential to a level far below that of oestrus. In some cases a reversal in polarity occurs at this point so that the vagina is positive to the symphysis pubis by a few hundred or even thousand microvolts. The electrical potential readings then remain low throughout the period of dioestrus. About forty-eight hours before an animal comes into oestrus, there is a marked rise in the potential in 58 per cent of the cycles studied (fig. 1, third cycle). This pre-oestrous rise is then followed by a drop before the normal oestrous peak occurs. The possible reasons for the failure to obtain a pre-oestrous rise in all the animals studied is discussed later in this paper.

While figure 1 is typical of the kind of curve obtained, the limits of variation are considerably greater than the graph indicates. This variation is clearly shown in a composite oestrous cycle made up of the individual

³ Throughout this paper the expressions "difference in potential" and "potential readings" have the same meaning; i.e., the difference in the number of microvolts between the two electrodes.

readings obtained (fig. 2). While the individual curve of each animal is similar to the average as shown in this figure, the magnitude of the potential readings varies considerably from rat to rat or even from cycle to cycle. Thus if the electrical determinations showed that the difference in potential of an animal at oestrus was 40,000 microvolts, the pre-oestrous readings of that cycle were high. On the other hand, a lower oestrous determination (20,000 microvolts) was always preceded by relatively small differences in potential. In both instances, therefore, the type of curve was the same.

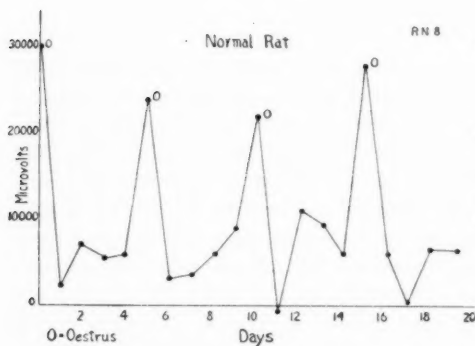


Fig. 1

Fig. 1. Difference in the electrical potential between the symphysis pubis and vagina in a typical normal rat. Readings taken daily for four oestrous cycles.

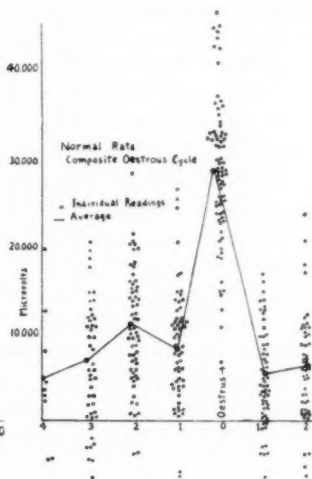


Fig. 2

Fig. 2. Composite picture of the difference in electrical potential during seventy oestrous cycles in twenty-eight normal rats.

Consistent results were obtained irrespective of the age of the animals. The twenty-eight rats of this series whose ages were known fell into two groups: one of about one hundred days, and the other two hundred. An average of the high oestrous readings of one age group exactly equalled that of the other. Similarly, the low oestrous average of the 100-day old rats was the same as that of the animals of two hundred days.

Eight animals were found to be in oestrus on two consecutive days. In each of these cases the potential reading was low on the first day and high on the second. From these results it is evident that the peak occurs late in oestrus rather than early. It will be shown later in this paper that when

an animal is kept in oestrus experimentally for more than one day, the oestrous rise occurs on the last day, just before the vaginal smear changes to one of post-oestrus.

Castrate animals. In order to determine the effect of castration upon the potentials, the ovaries were removed from twelve mature rats. Examination of three of these animals was deferred for two hundred and twenty-two days, while electrical potential readings were started on the others soon after castration. The animals of the first group were three hundred and sixty-five days old when readings were started. The remaining nine were divided into two groups according to age. The first was made up of four animals of about two hundred days, while the remaining five were over a year old. Thus the experiment is made up of three distinct groups:

1. Old, long time castrated rats
2. Moderately young, recently castrated rats
3. Old, recently castrated rats.

The type of curve obtained from plotting the daily readings was similar in all three groups. In each case the curve is quite patternless (fig. 3). A very high potential of one day may be followed by a low reading twenty-four hours later, which in turn may rise to a high peak the following day. The graph made from the potentials of one animal is quite different from all the others as far as the relationship of one reading to another is concerned; but similar in that the curve obtained in each case is patternless.

The readings of the older, long castrated rats are somewhat higher than those of the recently castrated old and young animals. In two rats the difference in potential was, on one or more occasions, well over 50,000 microvolts, which is higher than ever obtained in normal or other experimental animals. These results indicate that the length of time an animal has been ovariectomized and not the age of the rat determines the magnitude of the electrical potential.

Pseudopregnancy. During observation of the oestrous cycles of animals in the normal series, it was found that five were in a state of pseudopregnancy. Electrical determinations were made on these animals for a period of seven to twenty days in order to determine what effect this condition has on the normal electrical potential curve. The type of curve was found to be quite patternless and therefore similar in nature to that found in ovariectomized animals.

Ovariectomized rats receiving theelin. Having shown that, when plotted, the electrical potentials of a normal adult animal produce a pattern which can be definitely correlated with the oestrous cycle, and that removal of the ovaries changes the type of curve obtained to one which is entirely patternless, the next step was to determine whether or not the normal curve could be reproduced by theelin administration in ovariectomized

animals. For this purpose, sixty-three castrate rats were injected with varying amounts of aqueous theelin and theelin in oil.⁴ According to the amount of hormone given, the results fell into two groups: those animals receiving sub-threshold amounts of hormone, and those receiving enough to produce oestrus.

1. *Sub-threshold amounts of hormone.* Ten rats were injected with amounts of theelin which failed to bring them into oestrus. Five of these animals, given daily injections of 0.05, 0.1, 1 R.U. in water and 0.05 and 0.25 I.U. in oil respectively, had readings which were typically castrate in nature. The other five rats, given larger but still sub-threshold doses,

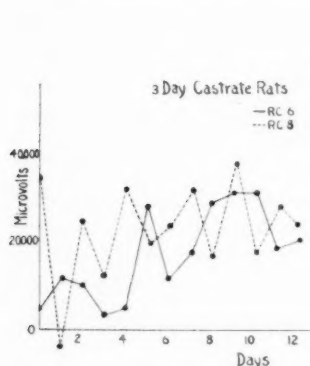


Fig. 3

Fig. 3. The daily electrical potentials of two ovariectomized rats. Note the patternless type of curve.

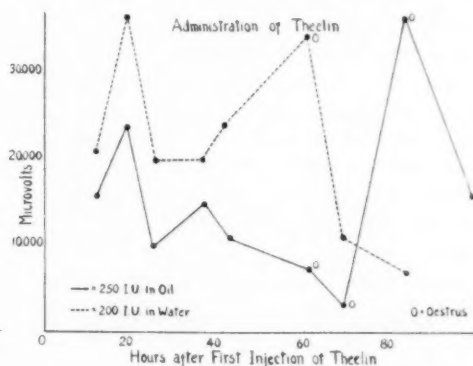


Fig. 4

Fig. 4. Comparison of the electrical potential curve obtained from ovariectomized rats given theelin in oil and aqueous theelin. Note that the oestrous rise in potential takes place at the end of the oestrous period.

gave an entirely different type of curve. After forty-eight hours the daily electrical determinations were practically identical so that, when plotted, the readings formed a fairly straight line. Two animals showing this flat type of curve received 5 and 2 R.U. of theelin in water per day for eight days but failed to come into oestrus.

These results indicate that very small amounts of theelin given in sub-cutaneous injections have no effect upon the potentials of rats, while larger subminimal doses tend to "iron out" the irregular castrate type of curve.

2. *Oestrous-producing amounts of theelin.* As it is impracticable to take

⁴ Kindly supplied by Parke, Davis & Co.

continuous readings on each rat from the time of the first injection of the hormone until after the animal has passed through oestrus, it was necessary to evolve a schedule of optimum times at which the electrical determinations should be made. The following time schedule was decided upon after many readings had been taken throughout the duration of the experiment:

DAY	TIME	NUMBER OF HOURS AFTER FIRST INJECTION OF HORMONE
1st	9 p.m.	0
2nd	9 a.m.	12
	4 p.m.	19
	9 p.m.	24
3rd	9 a.m.	36
	9 p.m.	48
4th	9 a.m.	60
	4 p.m.	67
	*9 p.m.	72
5th	*9 a.m.	84

* Readings were made at these times only when prolonged oestrus made it necessary.

It is felt that these readings are numerous enough to include all critical changes in the potential, at the same time making it unnecessary to subject the animal to more than three anesthetic doses of sodium amytal in one day.

Fifteen castrate animals were given injections of aqueous theelin in varying amounts and their electrical potentials taken according to the above schedule. In all cases the theelin was administered in three subcutaneous injections twelve hours apart. All animals came into oestrus and showed a decided potential rise similar to that of normal animals. Ten, 40, 100, and 200 I.U. were given in an effort to determine whether or not the height of the oestrous peak is affected by the amount of theelin administered. It was found that the rise in potential followed the all-or-none response; i.e., the magnitude of the reading had no relation to the amount of theelin given as long as it was sufficient to produce cornification.

Eleven animals (74 per cent) showed a pre-oestrous rise in potential occurring between twelve and nineteen hours after the first injection of theelin. These peaks may be similar to those previously noted in normal animals. However, in theelin-treated castrate rats, the problem is more complicated because of the irregular castrate baseline which must be over-

come by the action of the female sex hormone. Thus it requires about twelve to fifteen hours after theelin administration for the regular oestrous potential curve to supersede the irregular castrate type.

Nineteen rats were injected with from 10 to 250 I.U. of theelin in oil. Eighteen (95 per cent) of these animals showed the characteristic rise in potential. As in the experiments on rats receiving aqueous theelin, there was no relationship between the amounts of hormone given and the height of the oestrous peak. Only nine of the group showed a well-defined pre-oestrous peak such as was seen in 74 per cent of the animals injected with the aqueous solution.

Theelin given in oil, because of its slower absorption, keeps an animal in oestrus for a longer period than the same amount of hormone administered in an aqueous solution. The rise in potential occurs not at the beginning of oestrus, but just before the rat goes into post-oestrus. The similarities and differences in the type of curve obtained when theelin is given in water and in oil are shown in figure 4. A pre-oestrous rise took place at the nineteenth hour after the first injection of theelin. This was followed by a drop and comparatively low readings until the sixtieth hour, when both animals were found to be in oestrus. At this time, as shown by the graph, electrical determinations made on the rat receiving aqueous theelin showed a great increase in potential, while readings on the other animal remained low. Shortly after this oestrous peak, the rat receiving theelin in water went out of oestrus. Because of the slower absorption rate in oil, the second animal remained in oestrus until about the ninetieth hour. Its potential remained low until a few hours before post-oestrus set in, when the characteristic rise took place.

Prolonged oestrus. To determine whether or not the potential of an animal may be kept low during long periods of vaginal cornification, seven animals were kept in oestrus for from four to seven days by daily injections of 20 I.U. of theelin. During all but the last day of oestrus, the potential readings were low and fairly constant. On the day before each animal went out of oestrus, there was a rise in potential, followed by the usual drop at the time a post-oestrous smear was obtained.

Discussion. Site of electrode. The vagina and symphysis pubis were chosen as the most practical of the possible contact points for the electrodes even though higher potentials are obtained when the uterus is used instead of the symphysis pubis. The exterior end of a uterine fistula gives the same potential as the surrounding skin. Axilla to axilla, groin to groin, symphysis to rectum, have all been tried without success in reproducing the type of curve which, as determined by the experiments reported in this paper, is normal when the vagina and symphysis pubis are used. Burr (4) on the other hand, takes the difference in potential between the right and left fingers in determining the curve for the human menstrual cycle.

Anesthesia. It is necessary to anesthetize an animal before adjusting the electrodes. If an unanesthetized rat is strapped to a board and then given amytal while the potential readings are being taken, there is a slight decrease in the difference in potential after the animal ceases to struggle. Inasmuch as the determinations are relative rather than absolute, this slight reduction is of no importance.

Magnitude of the oestrous rise in potential. It was seen that the magnitude of the electrical potential reading in the normal rat varied considerably. Inasmuch as the complete rise and fall of the oestrous potential takes about fifteen hours, and that in these experiments determinations were taken but once a day, there was little possibility of obtaining the maximum difference in potential. This was shown to be so in several animals whose oestrous potentials were comparatively low at the usual time of making the determination. By retaking the electrical reading every few hours, a high peak in the electrical potential was eventually found in each case.

It will be recalled that only about 60 per cent of the rats showed a pre-oestrous rise in potential forty-eight hours before the main oestrous peak. It was found that this pre-oestrous rise is of even shorter duration than that of oestrus. Determinations made every few hours on several animals failing to show a pre-oestrous rise forty-eight hours before cornification demonstrated such a rise in each case later on during the second day preceding oestrus.

Those normal animals remaining in oestrus for more than one day showed the oestrous rise in potential on the second and not on the first day. Likewise, when castrate animals were kept in oestrus for varying lengths of time with theelin injections, the potential readings remained low until the last day of oestrus, when the characteristic rise took place. From this it may be seen that the rise in potential comes at the same time as the sloughing of vaginal epithelium and not during the growth or building up phase of the oestrous reaction.

SUMMARY AND CONCLUSIONS

1. During late oestrus in the normal rat there is a great increase in the difference in electrical potential between the vagina and the symphysis pubis. Comparatively low readings follow this peak until two days before the next oestrous period, when an increase in the number of microvolts takes place. This lesser pre-oestrous rise is then followed by a decrease in the potential which lasts until the late oestrous peak again occurs.

2. The cyclic type of electrical potential curve is abolished after removal of the ovaries. This is replaced by a castrate type of reading which, when plotted, forms a patternless curve. The graph of the readings of one ani-

mal is different from that of any other in that one cannot be superimposed upon the other, and similar in that all are of a patternless type.

3. The potentials of rats in pseudopregnancy are similar to those of ovariectomized animals.

4. Subcutaneous injections of very small quantities of theelin have no effect on the electrical potential of ovariectomized animals. Larger but still sub-threshold amounts cause the curve of potential readings to flatten out. If sufficient quantities of theelin are given to produce oestrus, the electrical determinations form a curve similar to that of the normal animal. Fifteen to twenty hours after the first of three injections there is a slight rise in potential. This is followed by low readings until a few hours before the rat goes out of oestrus, when a peak in the potential occurs. As soon as leucocytes appear in the vaginal smear, the difference in potential drops. Provided oestrus is induced, the type of curve and magnitude of the peak are the same irrespective of the amount of theelin given. When an animal is kept in oestrus for many days by injections of theelin, the potential readings remain low until just before post-oestrus sets in, when the oestrous rise takes place.

5. One concludes from these experiments that there is a definite electrical potential pattern associated with the oestrous cycle of the rat, that this disappears after ovariectomy or during pseudopregnancy, that the normal pattern of electrical potential readings may be reproduced by injection of theelin.

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VENOUS PRESSURE RESPONSES TO EXERCISE¹

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The most thorough studies on venous pressure during physical exercise have been reported by Hooker (3) and White (7) (8). Hooker found an increase in the venous pressure with muscular exertion which lasted throughout the work period and several minutes thereafter, while White found that with light exercise the increase in the venous pressure continued during the whole period but dropped to normal within 15 seconds after work was stopped. He also found that with heavy work the increase in venous pressure during the first several minutes was greater than with light exertion, but that it dropped back to, or below, normal before the end of the exercise period. This was followed by a further drop immediately after the work period. Since these results are not in agreement this present study was made in an attempt to determine more fully what changes take place in venous pressure throughout and immediately after a period of muscular activity.

White (6) is of the opinion that the disagreement between his results and those of Hooker might be accounted for by the fact that in each case a different method was employed to determine the pressure. In order to avoid this difficulty we used three methods: namely, the White (6) method, the Hooker (4) capsule, and Eyster's (1) modification of the original Hooker-Eyster (2) instrument.

All of our 19 subjects were men between 19 and 24 years of age. Each was required to ride a Krogh bicycle ergometer. Determinations of venous pressure were made at minute intervals during and after the period of exertion. In order to compare the 3 methods of determining venous pressure each subject on different days carried loads designated as light, moderate, and heavy; i.e., 4000, 6000, and 8000 ft. lbs. per minute. The period of work on the ergometer was 15 minutes.

We invariably found that readings obtained by the different methods, when plotted, gave similar graphs. The White procedure constantly gave readings somewhat higher than those of Hooker and Eyster. This was accounted for by the manner in which the end-point was determined. By

¹ The expense of this investigation has been met by a grant from the Charles Himrod Denison Fund.

the White method when the pressure is suddenly applied to the capsule, it is quickly raised from the surface of the skin and the skin under it is cupped. Because of this and since there is no gradual comparative change, it is exceedingly difficult to be certain of the pressure at which the vein shadow disappears. To remedy this the following procedure was used. When the vein is completely collapsed, there is a valley formed in the vein channel. This valley, with oblique lighting, shows up distinctly as a shadowed area. For this method, then, the end-point was taken the instant this valley, as indicated by the darkened region, could no longer be seen. With those subjects whose skin is very white and whose blood shows as a bluish color in the veins, it was found best to read the end-point according to the method of Krogh, Turner, and Landis (5). With the Hooker and Eyster methods the criterion of the end-point was the disappearance of the shadow cast by the vein as the pressure was gradually increased.

Venous pressure changes during a period of exercise. The type of response. The pressure rose in all experiments, conducted repeatedly with 19 subjects, and in none did it drop significantly during the period of exertion. As regards the type of response all the subjects can be divided into 2 distinct groups. In the first group composed of 8 men there was no "warming up" period. The venous pressure rose during the first minute and within 2 to 4 minutes reached a maximum which was then maintained fairly constant throughout the remainder of the exertion. In the other group of 11 subjects the pressure was slow in rising, in several no change appeared for several minutes and when the rise began it required 6 or more minutes to reach the maximum. The majority of this group reached the maximum in 10 or 12 minutes and then maintained a fairly steady state. The above differences in the 2 groups were most conspicuous when the light load of work was carried. With the heavy load the pressure began to rise in all cases during the first minute of exertion.

Composite curves for the 2 groups are given in figure 1. These were prepared by calculating the percentage of change in the venous pressure, as this method of comparison better lends itself for the blending of the individual cases into a composite curve.

Fatigue. For 2 of the subjects the 8000 ft. lbs. load was too heavy, since they reached a state of fatigue before the end of the 15-minute work period. The fatigue was localized in the legs, as they stated that their legs just would not go any longer and that they could not keep up with the beat of the metronome. One of these men served twice, the first time he worked 6 minutes during which his venous pressure rose steadily from 113 to 204 mm. H₂O, while the second time he held on for 7 minutes as the pressure rose from 121 to 230 mm. The second man continued to work for 9 minutes during which time the pressure rose from 128 to 215

mm. In none of the other subjects did the venous pressure rise as rapidly. Although we have no other supporting data, it may be conjectured that the heart was, in these cases, not compensating adequately to the demands of the occasion. At least the circulation through the legs was not enough to prevent the accumulation of lactic acid which, no doubt, caused the condition of local fatigue noted.

Relationship of load to venous pressure. Our early observations indicated that the venous pressure increased proportionately with each increment in the load of work. Hence 2 subjects were run through a series of 6 loads, with an interval of rest of 10 or more minutes between the work periods. These data are given graphically in figure 2. Since a steady state was

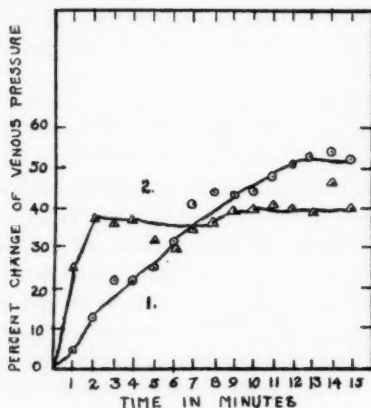


Fig. 1

Fig. 1. Venous pressure curves during a load of 4000 ft. lbs. 1. Average for 11 subjects. 2. Average, 8 subjects.

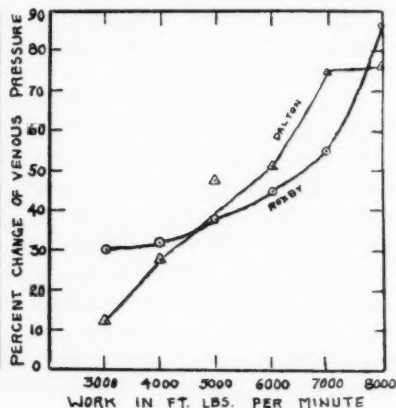


Fig. 2

Fig. 2. Linear relationship of load and venous pressure.

reached in all cases by the 12th minute of work, the mean of the last 4 readings was used in plotting the curve. It is evident that subject D increased his venous pressure in almost the same proportion as the increase in the load up to 7000 ft. lbs., where the curve flattens out.

In figure 3 we present a composite curve prepared by calculating the percentage, instead of using the actual, increase for each of 8 subjects who carried loads of 4000, 6000, and 8000 ft. lbs. Again the mean of the last 4 determinations was used, since by then the maximum increase had been reached. Although the curve obtained is not a straight line, the indication is, as shown later, that, if the inaccuracies of method could be removed and a constant condition obtained, the venous pressure rises in a linear relationship to the increase in load of work.

Influence of respiration on venous pressure. A factor that makes it difficult to establish a clear linear relationship between load and venous pressure is the variation in venous pressure with breathing. With deep breathing the venous pressure is higher during expiration than it is during inspiration. In figure 4 are given the curves obtained from an experiment with a load of 6000 ft. lbs. in which an attempt was made to make the readings of the venous pressure during each of the 2 phases of breathing. The inspiratory and expiratory venous pressures were most often between 1.5 and 2 cm. H₂O apart, with expiratory readings the higher. It is clear, therefore, that under the ordinary conditions of venous pressure determinations some irregularity in pressures will be recorded even after a steady physiological state has been reached.

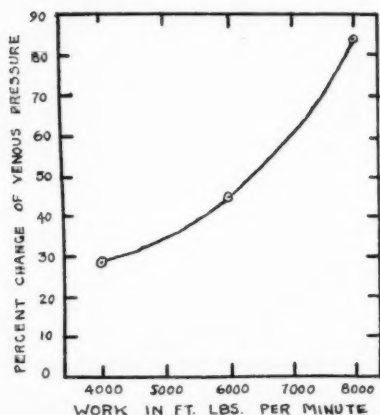


Fig. 3

Fig. 3. Composite curve from 8 subjects.

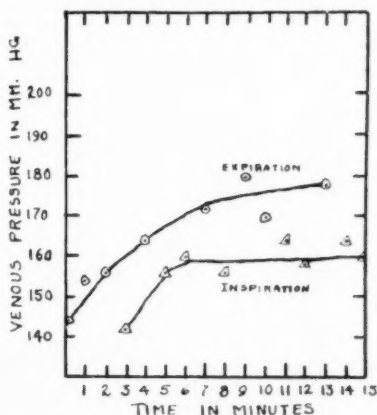


Fig. 4

Fig. 4. Influence of respiration on venous pressure during a load of 6000 ft. lbs.

The after effect of exercise on venous pressure. Recovery from the effects of exercise was ordinarily followed for only 10 minutes; but in 7 cases, after a load of 8000 ft. lbs. had been carried for 15 minutes, the return was followed for 30 minutes. In none of the men was a subnormal level observed at any time during the period of recovery. Also, contrary to White's observations, none of our subjects, with any of the loads, experienced a return to normal venous pressure immediately after the cessation of the exertion. In one exceptional subject, after a load of 4000 ft. lbs., recovery was made by the end of the first minute; but even this man required 7 minutes for recovery after carrying a load of 6000 ft. lbs, and 8 minutes after a load of 8000 ft. lbs.

After the heavy load of work, 8000 ft. lbs., the curves for the return of

the pressure were of 2 types—see figure 5. The members of one group (A) experienced during work an average pressure rise of 60 per cent, which dropped back to normal within 6 to 9 minutes of rest. The other group (B) averaged an 86 per cent increase in pressure and did not return to the normal level until the 22nd to 27th minutes. The most rapid fall in pressure for each group occurred during the first 5 minutes. During that interval group A very nearly returned to normal, while group B showed only a moderate drop.

Among the 9 men who carried the 3 loads of work—light, moderate, and heavy—there were 3 who failed to recover within 10 minutes after

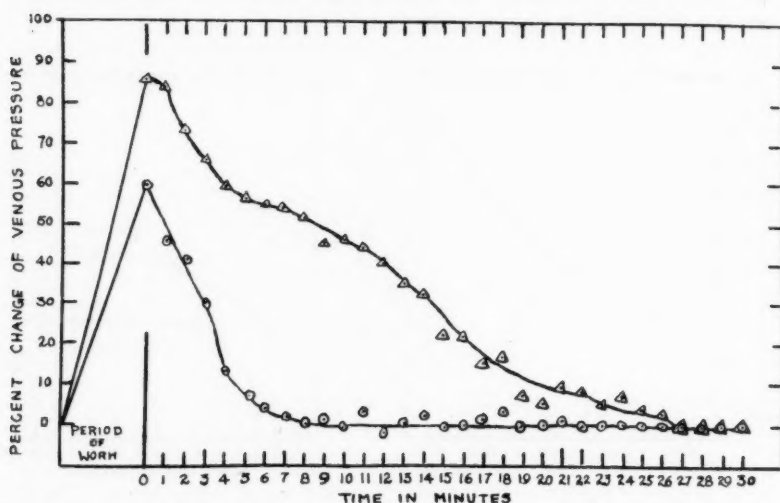


Fig. 5. Curves for recovery of venous pressure after a load of 8000 ft. lbs. for 15 minutes.

any of the loads. All of the others showed a lengthening of the period of recovery with each successive increase in the size of the load.

SUMMARY

A comparison of the Eyster, Hooker, and White methods of determining venous pressure shows that each gives the same account of the changes that result from physical exertion.

The venous pressure rises and then remains up during work on the bicycle ergometer. In some individuals the pressure begins to rise almost at once, reaches a maximum within 2 to 4 minutes, and then maintains a fairly steady state. In others after some delay a slow rise begins, reaches a maximum within 10 to 12 minutes, and then remains fairly constant until work is terminated.

When the load of work is too heavy the venous pressure rises steadily until fatigue ensues.

There is a rough linear relationship between venous pressure and load. This may be obscured by the deep breathing of exertion, since during expiration the venous pressure may be as much as 2 cm. H₂O higher than during inspiration.

After physical exertion the venous pressure ordinarily slowly returns to normal. This may be accomplished within a few minutes, but after heavy work often requires as much as 22 to 27 minutes.

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THE FORMATION AND BEHAVIOR OF COLLOIDAL CALCIUM PHOSPHATE IN THE BLOOD¹

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Following the suggestion of Eichholtz and Starling (1) that under certain conditions a non-diffusible colloidal complex of calcium and phosphate might be formed in the blood, it is now well established that such a substance may be made to appear in the plasma, *in vivo* or *in vitro*, by augmenting either the calcium or the phosphate concentration, or both. The literature concerning this phenomenon has recently been reviewed by Schmidt and Greenberg (2). The exact composition of the compound is not known, and little is known as to its significance and behavior in the animal organism.

Binger (3) administered large doses of phosphates intravenously to animals, and observed a fall in serum calcium, accompanied by tetany when the phosphate solution used was neutral or alkaline. Salvesen, Hastings and McIntosh (4) observed a fall in serum calcium and tetany following oral administration of phosphates. The present investigations were undertaken in order to study the rôle of the formation and disposal of the colloidal complex of calcium and phosphate in these phenomena.

Previous investigations concerning colloidal calcium phosphate have been carried out by means of dialysis or ultrafiltration. The present investigations, which have depended upon direct observation of Ca^{++} concentrations, and upon the mass law relationship between calcium and protein previously described (5) have disclosed an important time factor in the reaction between calcium and phosphate, not accessible to diffusion methods. The observations reported have accordingly thrown new light upon this reaction, as well as upon the behavior of its product in the organism. The quantitative results are however subject to the reservation that under the conditions of the experiments the ionic strength of the fluids examined was made to vary widely, and that consequently the Ca^{++} concentrations reported must for the present be regarded as of relative rather than absolute significance.

METHODS. Total calcium was determined by the method of Kramer

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and Tisdall (6) as modified by Clark and Collip (7). Total nitrogen was determined by the Kjeldahl method. Total protein of the plasma was calculated from total nitrogen less non-protein nitrogen, using an arbitrary correction of 30 mgm. per 100 cc. for the latter and a factor of 6.3 to convert grams of nitrogen to grams of protein. Observed Ca^{++} concentrations were obtained by the frog-heart method (8). Sodium polyanetholsulphonate was used as an anticoagulant, and initial observations of Ca^{++} concentration in plasma were made by the use of whole blood, as previously described (9). Inorganic phosphate was determined by the method of Fiske and Subbarow (10). Calcium bound to protein was calculated from Ca^{++} and total protein concentrations, using the nomogram previously published (5). The difference between total calcium and that accounted for as Ca^{++} and as calcium bound to protein was designated as CaX and assumed to represent colloidal calcium phosphate.

Use has been made of an empirical solubility product, $\text{Ca}^{++} \times \text{P} = \text{K}$, in which both factors are expressed in millimols per liter and P represents total inorganic phosphate. Use of this product depends upon the assumption that the concentration of the ion reacting with Ca^{++} is proportional to the concentration of total phosphate. The product further is theoretically valid only if the Ca^{++} and the corresponding phosphate ion combine with each other in a 1:1 ratio. Use of this product, based upon the empirical $\text{Ca} \times \text{P}$ product of Howland and Kramer (11) appears to afford information of some value.

EXPERIMENTAL. *Addition of phosphate to serum in vitro.* An M/10 mixture of Na_2HPO_4 and NaH_2PO_4 , adjusted to pH 7.4, and approximately isotonic with human serum, was added to serum in amounts sufficient to bring the phosphate concentration to the desired level. The mixture contained 75 per cent serum, the required amount of the phosphate mixture, and 0.9 per cent NaCl to volume, the phosphate being added immediately before observations were begun.

The results as shown in figure 1, which includes observations on one sample of serum, indicate that the Ca^{++} concentration falls when sufficient phosphate is added, the fall continuing until equilibrium is reached. Both the rate of fall in Ca^{++} concentration and the level at equilibrium are shown to be dependent upon the concentration of phosphate present.

These results are clearly due to the formation of a physiologically inactive combination of calcium with phosphate, presumably colloidal in character. The $\text{Ca}^{++} \times \text{P}$ product at equilibrium apparently approaches constancy at about 3.0, less reliance being put on the absolute value of Ca^{++} concentrations observed in the presence of 25 mM of phosphate. This is in apparent contradiction to the results obtained on adding phosphate to casein solutions containing calcium, as reported by McLean and Hastings (5, p. 312) in which case the addition of 10 mM of phosphate was

followed by no appreciable fall in Ca^{++} concentration. The difference is in part due to failure to take account of the time factor in the previous experiments and in part to the fact that the reaction between calcium and phosphate takes place much less rapidly in solution of casein than in serum. The nature of this difference is being further investigated.

Intravenous administration of phosphate to dogs. In the first series of experiments dogs were subjected to large doses of an M/2 mixture of Na_2HPO_4 and NaH_2PO_4 adjusted to pH 7.4, injected intravenously, and the changes in the blood were followed over a period of 5 to 8 hours. The dosage employed corresponded to 150 mgm. of phosphorus per kilogram of body weight, the amount found by Binger (3) to produce the characteristic

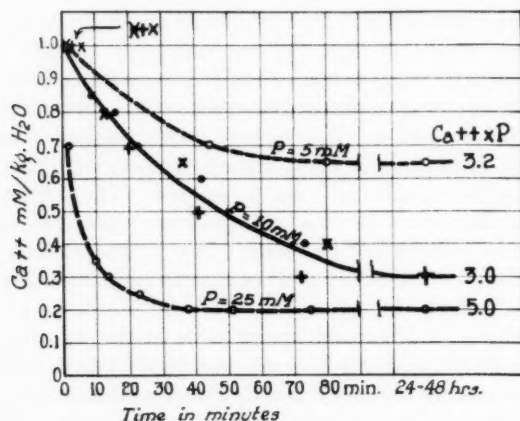


Fig. 1. Rate of fall in Ca^{++} concentration and equilibrium conditions in normal human serum following addition of phosphate.

effects. It will be noted that certain of the immediate effects following administration of this solution are to be attributed to its hypertonicity.

In the early experiments, in which serum was examined after coagulation and separation of blood, it was found that a further fall in Ca^{++} concentration took place when the serum was allowed to stand over night. Consequently the method previously described (9) for examination of whole blood was applied within 2 to 3 minutes after withdrawal of blood from the animals, and plasma, after separation, was used for observations up to 24 hours. Complete experiments then include observation of the course of events in the animal, based upon observations immediately following withdrawal of blood, and of the further changes in plasma outside of the animal. The protocol of such an experiment from which are omitted all except the initial and final observations of Ca^{++} concentrations, is shown

in table 1. The final Ca^{++} concentrations were observed 24 hours after withdrawal of blood.

Table 1 illustrates the following significant findings, characteristic of all similar experiments. Within two minutes from the end of an intravenous injection of a large dose of phosphate there is a marked rise in the phosphate concentration of the plasma, and a decrease in the total calcium concentration, the latter attributable chiefly to dilution. The Ca^{++} concentration has fallen to a low level, and approximately two-thirds of

TABLE 1
Intravenous administration of phosphate

Dog, weight 8.2 kgm., injected intravenously with 82 cc. M/2 phosphate mixture, 11:09-11:13 a.m. Convulsive movements during injection. Stiffness of forelegs noted until 11:25. Twitching of muscles, 11:45-2:05. Generalized convulsion 4:50 p.m., during withdrawal of blood. Symptoms relieved by calcium gluconate, following which dog survived.

SAMPLE NUMBER	TIME			OBSERVED					CALCULATED					
	Blood drawn	Initial Ca^{++} observation	Elapsed from end of injection	Plasma protein	Plasma phosphate	Plasma total Ca	Plasma Ca^{++}		Initial			Final		
							Initial	Final	CaProt	CaX	$\text{Ca}^{++} \times \text{P}$	CaProt	CaX	$\text{Ca}^{++} \times \text{P}$
			minutes	gm. per 100 cc.	mM per kgm. H_2O	mM per kgm. H_2O	mM per kgm. H_2O	mM per kgm. H_2O	mM per kgm. H_2O	mM per kgm. H_2O		mM per kgm. H_2O	mM per kgm. H_2O	
1	10:38			5.7	1.1	2.49	[1.25]*	[1.25]*	1.24		1.4	1.24		1.4
2	11:15	11:18	2	4.6	31.3	1.84	0.3	0.1	0.27	1.27	9.4	0.10	1.64	3.1
3	11:34	11:37	21	6.1	12.6	2.32	0.5	0.2	0.60	1.22	6.3	0.25	1.87	2.5
4	12:15	12:17	62	5.6	6.3	1.82	0.7	0.5	0.75	0.37	4.4	0.55	0.77	3.1
5	2:14	2:18	181	5.5	4.9	2.03	0.8	0.7	0.82	0.41	3.9	0.73	0.60	3.4
6	4:48	4:52	335	5.9	2.4	2.17	0.9	0.9	0.98	0.29	2.2	0.98	0.29	2.2

* The brackets [] indicate that this value was calculated from total calcium and total protein.

the total calcium is calculated to be in the form of colloidal calcium phosphate. (The absolute value for Ca^{++} concentration, and accordingly the validity of the calculations from this value, are in doubt, owing to the increase in ionic strength following administration of a hypertonic phosphate solution. Presumably the Ca^{++} concentration is somewhat higher than shown.) The Ca^{++} concentration continued to fall after withdrawal of blood, until equilibrium was reached.

After 20 minutes there is a fall in the phosphate concentration, and a decrease in the total calcium concentration, not due to dilution. The Ca^{++} concentration has risen, and there is still a high concentration of colloidal

calcium phosphate. The Ca^{++} concentration in the plasma continued to fall after withdrawal of blood. After one hour there is a further fall in phosphate and in total calcium, a rise in Ca^{++} , and a sharp decrease in colloidal calcium phosphate. Again the Ca^{++} concentration decreased in the sample of blood following its withdrawal. At 3 hours and at $5\frac{1}{2}$ hours, the evidences of recovery predominate, with increasing total calcium and Ca^{++} , and with little or no decrease in Ca^{++} concentration in the samples of blood following withdrawal.

Inspection of the $\text{Ca}^{++} \times \text{P}$ products shows that when the product exceeded approximately 3.0 on withdrawal of blood the Ca^{++} concentration

TABLE 2
Oral administration of phosphate

Dog, weight 8.8 kgm., given 100 cc. m/2 phosphate mixture at approximately hourly intervals from 9:35 a.m. to 3:07 p.m.; total 600 cc. Thirst, vomiting and diarrhea appeared after 12:30. Depressed from 1:30. Generalized twitchings, especially hind legs, at 3:42. Symptoms relieved by calcium gluconate after final blood sample, following which dog survived.

SAMPLE NUMBER	BLOOD TAKEN	OBSERVED					CALCULATED					
		Plasma protein	Plasma phosphate	Plasma total Ca	Plasma Ca ⁺⁺		Initial			Final		
					Initial	Final	CaProt	CaX	Ca ⁺⁺ × P	CaProt	CaX	Ca ⁺⁺ × P
	time	gm. per 100 cc.	mM per kgm. H ₂ O	mM per kgm. H ₂ O	mM per kgm. H ₂ O	mM per kgm. H ₂ O	mM per kgm. H ₂ O	mM per kgm. H ₂ O		mM per kgm. H ₂ O	mM per kgm. H ₂ O	
1	9:23	6.8	0.91	2.58	[1.15]*	[1.15]*	1.43		1.0	1.43		1.0
2	11:48	5.3	3.04	2.24	0.9	0.9	0.88	0.46	2.7	0.88	0.46	2.7
3	1:55	5.0	4.63	1.99	0.65	0.6	0.63	0.71	3.0	0.58	0.81	2.8
4	3:55	5.2	3.62	1.81	0.65	0.65	0.65	0.51	2.3	0.65	0.51	2.3

* The brackets [] indicate that this value was calculated from total protein and total calcium.

continued to fall until equilibrium was reached, but that no further change occurred in the blood *in vitro* when the product was below 3.0.

It is apparent that three processes take place simultaneously during the experiment. Colloidal calcium phosphate is formed in the plasma, it is removed from the blood, and calcium in solution is returned to the blood. The findings at any particular time are the resultant of these three processes. It is significant that the greatest formation of colloidal calcium phosphate is within the first twenty minutes after injection, and that after one hour it is present in the blood only in small amounts.

Oral administration of phosphate. In a second series of experiments the m/2 phosphate mixture was administered orally in amounts corresponding

to those given by Salvesen, Hastings and McIntosh (4). All of the dogs receiving this mixture by stomach tube exhibited thirst, vomiting and diarrhea, so that the amount absorbed was much less than that administered. A protocol of such an experiment is given in table 2.

The data from this and similar experiments indicate that colloidal calcium phosphate is formed in and removed from the blood, as in the case of a single large intravenous injection, the differences in the findings being attributable to the method of administration. Little or no change in the Ca^{++} concentration in the plasma following withdrawal of blood has been noted, indicating that equilibrium had already been attained *in vivo*, a conclusion also supported by the empirical $\text{Ca}^{++} \times \text{P}$ products found.

TABLE 3

Intravenous administration of colloidal calcium phosphate

Normal dog, weight 6.9 kgm. Forty-five cubic centimeters horse serum + 45 cc. $\text{M}/10 \text{ CaCl}_2$ mixed with 30 cc. horse serum + 30 cc. $\text{M}/10$ phosphate mixture and injected immediately. No reaction to injection and no symptoms during period of experiment, except that dog refused food during last three hours.

SAMPLE	ELAPSED TIME FROM INJECTION	OBSERVED				CALCULATED			
		Plasma protein	Plasma phos- phate	Plasma total Ca	Whole blood total Ca	Excess Ca	Excess P	Excess Ca Excess P	Ca whole blood Ca plasma
	hours	gm. per 100 cc.	mM per l.	mM per l.	mM per l.	mM per l.	mM per l.		
1	Control	5.8	1.13	2.75	1.72				0.63
2	0	4.7	2.59	5.44	3.48	1.46	2.69	1.84	0.64
3	$\frac{1}{2}$	5.2	1.81	4.16	2.74	0.68	1.41	2.07	0.66
4	1	5.6	1.43	3.95	2.45	0.30	1.20	4.00	0.62
5	2	5.2	1.30	2.93	1.82	0.17	0.18	1.06	0.62
6	4	4.9	1.17	2.79	1.87				0.67

Intravenous administration of colloidal calcium phosphate. Preformed colloidal calcium phosphate, suspended in horse serum, was administered intravenously to two normal dogs and to one thyroparathyroidectomized dog in a state of chronic latent tetany without symptoms and without treatment. Protocols of two of these experiments are included in tables 3 and 4.

Both of these experiments indicate a rapid removal of the added calcium and phosphate from the blood, the removal being more rapid in the case of the normal dog. It was hoped that the ratios of excess Ca to excess P in the plasma might give some clue to the composition of the colloidal complex, but the results were too irregular to be of any value in this respect. Table 4 indicates that at $\frac{1}{2}$ and $1\frac{1}{2}$ hours after injection the excess calcium

remaining in the blood was all in the form of the colloidal compound, the calculated values for CaX being higher than the values for excess Ca, but the ratio of excess Ca to excess P corresponds neither to $\text{Ca}_3(\text{PO}_4)_2$ nor to CaHPO_4 .

Because of the possibility that the colloidal complex might be adsorbed upon the red blood cells, and in order to determine whether or not the excess Ca was actually being removed from the blood, total Ca determinations on whole blood were made in one experiment (table 4). These analyses, and the ratios of Ca in whole blood to Ca in plasma, indicate that no adsorption on red blood cells had taken place.

TABLE 4

Intravenous administration of colloidal calcium phosphate

Dog, weight 8.4 kgm., in state of chronic latent tetany, without treatment, following thyro-parathyroidectomy. Thirty-seven and five-tenths cubic centimeters horse serum + 37.5 cc. M/10 CaCl_2 mixed with 25 cc. horse serum + 25 cc. M/10 phosphate mixture and injected immediately. No reaction to injection and no symptoms during period of experiment.

SAMPLE NUMBER	ELAPSED TIME FROM INJECTION	OBSERVED					CALCULATED				
		Plasma protein	Plasma phosphate	Plasma total Ca		Plasma Ca^{++}	CaProt	CaX	Excess P	Excess Ca	Excess Ca Excess P
	hours	gm. per 100 cc.	mM per l.	mM per l.	mM per kgm. H_2O	mM per kgm. H_2O	mM per kgm. H_2O	mM per kgm. H_2O	mM per l.	mM per l.	
1	Control	6.8	1.62	1.77	1.89	0.7					
2	0	5.2	2.79	3.39	3.57	*			1.17	1.62	1.38
3	$\frac{1}{2}$	6.0	2.50	2.77	2.93	0.8	0.9	1.2	0.88	1.00	1.14
4	$1\frac{1}{2}$	5.4	2.18	2.47	2.60	0.8	0.8	1.0	0.56	0.70	1.25
5	$3\frac{1}{2}$	6.2	1.84	2.26	2.39				0.22	0.49	2.22

* Ca concentration above limit of sensitivity of frog's heart.

DISCUSSION. A qualitative interpretation of these experiments offers, within certain limits, no difficulty. Augmentation of the phosphate concentration of the plasma, by whatever route, leads, as shown by others, to formation of colloidal calcium phosphate. The formation of this substance at the expense of Ca^{++} and calcium bound to protein is a function of Ca^{++} and phosphate concentrations, and of time. It quickly disappears from the plasma, resulting in a lowering of the total calcium, and explaining the failure of McLean and Leiter (12) to find evidence of its accumulation in the blood in conditions in which its formation was to be expected. In the intact animal recovery, associated with a return of calcium in solution to the plasma, occurs *pari passu* with the progress of the reaction between

calcium and phosphate. The mechanism of removal of colloidal calcium phosphate from the blood has been studied by Dr. I. Gersh, whose results are reported in an accompanying paper (13).

The occurrence of tetany would appear to be related to the fall in Ca^{++} concentration in the plasma, but as in the case of tetany following parathyroidectomy (9) its appearance and severity are not directly correlated with the Ca^{++} level, thus suggesting the need for consideration of other variables.

The quantitative aspects of the experiments are not at present capable of full interpretation. Making due allowance for the questionable accuracy of the method for observation of Ca^{++} concentration, under the conditions of these experiments, and for the necessity for using an empirical $\text{Ca}^{++} \times \text{P}$ product, the results suggest that the formation of colloidal calcium phosphate depends, as was to be expected, upon the solubility of a difficultly soluble compound. The product obtained is of the order of magnitude of the solubility product obtained by Shear, Washburn, and Kramer (14) for CaHPO_4 . There is no further evidence that this represents the composition of the colloidal substance, and such evidence as there is (4) (15) is that it is $\text{Ca}_3(\text{PO}_4)_2$. The similarity of the $\text{Ca}^{++} \times \text{P}$ product to that found for CaHPO_4 is consistent with the suggestion advanced by Wendt and Clarke (16) that formation of CaHPO_4 is a first step in the precipitation of $\text{Ca}_3(\text{PO}_4)_2$.

The experiments as reported do not warrant further speculation as to the nature of the compound, or closer definition of the conditions for its formation. They indicate clearly, however, the necessity of taking the colloidal compound of calcium and phosphate into consideration in any studies concerning the solubilities of these substances in the presence of each other and in the presence of a protective colloid.

SUMMARY

1. When phosphate is added to serum *in vitro* it combines with calcium to form a physiologically inactive compound (shown by others to be colloidal in nature). A time factor in this reaction has been demonstrated.

2. When phosphate, at the pH of the plasma, is administered intravenously to dogs in doses sufficient to cause tetany, there is rapid formation of the colloidal compound, at the expense of Ca^{++} and calcium bound to protein, and rapid disappearance of this substance from the blood. The reaction between calcium and phosphate continues *in vitro* after withdrawal of blood until equilibrium is reached.

3. When phosphate is administered orally to dogs in doses sufficient to cause tetany similar phenomena are observed, the differences being attributable to the rate of absorption of phosphate.

4. Tetany occurs following administration of phosphate only when the Ca^{++} concentration in the plasma is reduced. Neither its occurrence nor its severity can be further correlated with the Ca^{++} concentration.

5. When preformed colloidal calcium phosphate, suspended in serum, is administered intravenously it is rapidly removed from the blood of both normal and thyroparathyroidectomized dogs.

6. No indication of adsorption of colloidal calcium phosphate on red cells has been found.

7. Following administration of phosphate *in vivo* and addition of phosphate to serum *in vitro* the product $\text{Ca}^{++} \times$ total inorganic phosphate (both expressed in millimols per liter) approaches a constant value of approximately 3.0 at equilibrium. When blood is withdrawn from the animal before equilibrium is reached the reaction continues to equilibrium *in vitro*.

8. It is suggested that the product $\text{Ca}^{++} \times \text{P} > 3.0$ represents the conditions for the formation of colloidal calcium phosphate in plasma.

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THE FATE OF COLLOIDAL CALCIUM PHOSPHATE IN THE DOG

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In the preceding paper McLean and Hinrichs¹ describe the formation and behavior in the circulating blood of a colloidal calcium phosphate. This compound is rapidly removed from the blood plasma and its existence even when present in massive amounts, is rather transitory. In this report, some experiments on a mechanism for this rapid removal of the colloidal substance from the blood are described. Evidence is presented that the colloidal particles of calcium phosphate are phagocytized by the macrophages of the liver and spleen. As the calcium and phosphate concentrations of the blood plasma are reduced in the natural course of events, the phagocytized calcium and phosphate are returned to the blood. The existence of the granules of calcium phosphate in the cytoplasm of the phagocytes, then, is also transitory. The macrophages of the lymph nodes take no part in this process. The reasons for their refractoriness and at least some of the conditions under which they may become active are analyzed in some additional experiments.

MATERIAL AND METHODS. The experiments reported here were all performed on dogs. Similar studies have been made on rats with similar results. They will be published separately in a current number of the *Anatomical Record*. Each series of dogs used in a single experiment were all fed with the same diet at the same time. Except in series I, table 2, all animals were accustomed to eat bread prepared in loaves from corn meal and liver ground together and baked. They were injected intravenously with a 5 per cent solution of hydrated calcium chloride, a 5 per cent solution of dibasic sodium phosphate neutralized with a solution of monobasic sodium phosphate, or with preformed colloidal calcium phosphate (3:2) suspended in horse serum. The dogs were killed at stated intervals after the completion of the injection by bleeding at the neck while under ether anesthesia. Small pieces of the liver, spleen, brachial lymph nodes and red bone-marrow (from the femur) were removed as rapidly as possible and frozen in liquid air or nitrogen. The tissue-blocks were dried in a vacuum at $-30^{\circ}\text{C}.$, embedded in paraffin and sectioned. Sections were tested histo-chemically for phosphate with a silver reagent

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and for calcium with alizarine by methods described in the paper referred to above. The localization of these two substances was so nearly identical in a few trial sections that it was believed to be justifiable to regard the black granules observed after the application of the silver reagent and representing largely or entirely phosphate as calcium phosphate.

RESULTS. *Normal, uninjected dogs.* Three dogs that were fed on the stock diet were anesthetized with ether and killed by bleeding. The macrophages of the liver and spleen show no trace of phagocytic activity. A fourth dog was deprived of this food for one day and then sacrificed. A very few scattered Kupffer cells of the liver contained a small number of

TABLE 1

Table showing the general course of the process of phagocytosis of colloidal calcium phosphate by the macrophages of the liver and spleen of the dog after the intravenous administration of calcium or phosphate solution injected separately in different animals or of both injected in succession in the same animal

BODY WEIGHT	ETHER	DOSAGE OF Ca AND P	TIME OF INJECTION	TIME AFTER INJECTION	DEGREE OF PHAGOCYTOSIS IN	
					Liver	Spleen
<i>kgm.</i>		<i>mgm. per kgm. body weight</i>	<i>minutes</i>	<i>minutes</i>		
11	+	20 P	3	Immediately	+	+
5.1	+	20 P	2	10	++	+
11	-	20 P	2	30	+	0
11	-	20 P	2	90	+	++
10	+	45 Ca	7	7	+	++
10.5	+	45 Ca	6	15	++	+++
8	+	45 Ca	3½	90	tr.	+
9	+	15 P	1	1	++++	++++
		27 Ca	2			
10	+	12.5 P	½	30	0	++++
		22.5 Ca	½			

granules of calcium phosphate. There was no evidence of activity by the phagocytes of the spleen in the same animal.

Effect of an intravenous administration of soluble salts of calcium or phosphate injected alone or successively on the phagocytosis of calcium phosphate (table 1). Under the conditions of the experiment, colloidal calcium phosphate is formed in the blood plasma. It is engulfed by the macrophages of the liver (Kupffer cells) and spleen (the littoral cells, the free and fixed macrophages of the red pulp). The degree of phagocytosis is estimated and represents a measure of the number of cells which are active in this process and the number of granules ingested by them. After the injection of either soluble salt alone the process of phagocytosis begins directly after the colloidal compound is formed in the plasma. The degree of phagocytosis is very marked at first (10-15 min.). In the course of

1½ hours the calcium phosphate present in the macrophages in the form of granules is slowly liberated. The degree of phagocytosis is more marked when both salts are injected in rapid succession. The macrophages of bone marrow and lymph node do not engulf the colloidal compound.

Effect of an intravenous administration of colloidal calcium phosphate on the phagocytosis of this compound (table 2). The two series of dogs give a markedly consistent picture of the course of the process of phagocytosis of the colloidal compound and of its liberation from the macrophages. The particulate material is rapidly engulfed, the process reaching a peak

TABLE 2

Table showing the general features of the removal of colloidal calcium phosphate from the blood by the phagocytes of the liver and spleen

The colloidal calcium phosphate was suspended in horse serum just preceding the injection. The calcium phosphate is phagocytized in the two organs rapidly during and after the injection, and is released more slowly into the blood stream in the course of 150 minutes. The dogs in series I were fed with the standard bread supplemented liberally by fresh meat and liver for three days preceding the experiment. The dogs in series II were fed with the bread alone. The injection of all animals with the colloidal calcium phosphate solution was completed in 4 to 5½ minutes.

SERIES	BODY WEIGHT	ETHER	DOSAGE OF $\text{Ca}_3(\text{PO}_4)_2$	TIME AFTER INJECTION	DEGREE OF PHAGOCYTOSIS IN	
					Liver	Spleen
	<i>kgm.</i>		<i>cc. per kgm. body weight</i>	<i>minutes</i>		
I	6.3	+	15	Immediately	+++	0
I	4.8	+	15	15	+++++	+++++
I	7.5	—	15	30	++	+++
I	6.6	—	15	60	+	++
I	6.8	—	15	150	0	+
II	5.5	+	13	Immediately	++	++
II	8+	—	13	15	+	+++++
II	8+	—	13	30	0	+++
II	7.2	—	13	60	0	++
II	7	—	13	150	0	+

in fifteen minutes. After this time the amount of colloidal calcium phosphate remaining in the cells slowly declines to the point where 1½ hours after the injection, there is only a scant retention of this compound. Although the Kupffer cells of the liver appear to be most active in the process of phagocytosis, they are also the first to liberate their ingested calcium. There is no evidence that the macrophages of bone marrow and lymph node play any rôle in the phagocytosis of this compound.

Effect of a subcutaneous and intramuscular injection of a soluble phosphate or calcium salt or of preformed colloidal calcium phosphate (table 3). After a subcutaneous and intramuscular injection of each of these three sub-

stances in the left foreleg and shoulder, the free and fixed macrophages of the brachial lymph nodes on the same side phagocytize the colloidal compound to various degrees. The corresponding lymph nodes on the opposite side give no indication of phagocytic activity for the particulate substance. When either of these two soluble salts is injected subcutaneously and

TABLE 3

Table demonstrating that after the subcutaneous and intramuscular injection into the left foreleg and shoulder of soluble phosphate or calcium, or of colloidal calcium phosphate suspended in horse serum, colloidal calcium phosphate is phagocytosized by the free and fixed phagocytes of the brachial lymph nodes on the same side draining the area.

Phagocytes in lymph nodes on the opposite uninjected side give no evidence of such phagocytosis. The degree of phagocytosis is enhanced when, in addition to such a subcutaneous and intramuscular injection of the left foreleg and shoulder with soluble phosphate or calcium solutions, it is preceded or followed in the same animal by intravenous injections of calcium or phosphate solutions respectively. Phagocytes in brachial lymph nodes on the opposite uninjected side again give no evidence of any activity. The time between the subcutaneous and intravenous injection is five minutes. The phosphate is injected as a 4.6 per cent solution, the calcium as a 5 per cent solution. In the double injections the order of injection is indicated by the numbers. All animals were anesthetized with ether during the injection.

INJECTION OF	BODY WEIGHT	TIME AFTER END OF LAST INJECTION	DEGREE OF PHAGOCYTOSIS IN LYMPH NODE
	kgm.	minutes	
40 cc. P subcutaneously and intramuscularly.....		105	+++
40 cc. Ca subcutaneously and intramuscularly.....		180	+
40 cc. Ca subcutaneously and intramuscularly.....		90	++
55 cc. $\text{Ca}_3(\text{PO}_4)_2$ subcutaneously and intramuscularly..		105	+++
(1) 25 cc. Ca intravenously.....	5	90	++++
(2) 40 cc. P subcutaneously and intramuscularly.....			
(1) 40 cc. P subcutaneously and intramuscularly.....	6	90	++++
(2) 25 cc. Ca intravenously.....			
(1) 60 cc. P intravenously.....	5	40	++++
(2) 40 cc. Ca subcutaneously and intramuscularly.....			
(1) 40 cc. Ca subcutaneously and intramuscularly.....	9	105	++++
(2) 72 cc. P intravenously.....			

intramuscularly into the left foreleg and shoulder and is preceded or followed by a vascular injection of the other salt, the degree of phagocytosis in the left lymph nodes is appreciably increased. In these cases also there is no visible evidence of ingestion of calcium phosphate by the macrophages of the brachial lymph nodes on the uninjected side.

DISCUSSION. In the normal dog no colloidal particles of calcium phosphate are present in the blood plasma. Hence it is not surprising that

the phagocytes of the liver and spleen contain no intracellular granules of this compound. The reason for the presence of some phagocytized granules in a few Kupffer cells of the normal dog deprived of food for one day is still to be investigated.

The chemical studies by McLean and Hinrichs confirm the concept that after the intravenous injection of large amounts of sodium phosphate into normal dogs, a physiologically inactive, colloidal calcium phosphate is formed in the blood stream. Coincidentally, there is a fall in the calcium ion concentration and in the calcium bound to the plasma proteins. They show also that this colloidal compound disappears rapidly from the blood, resulting in a decrease in the total plasma calcium. These results are caused at least in part by the abstraction from the blood plasma by phagocytosis of the colloidal particles and their temporary storage in the macrophages of the liver and spleen. The same process takes place after injections of calcium chloride or after the successive introduction of calcium chloride and sodium phosphate in the same animal. The colloidal particles of calcium phosphate are retained in the cytoplasm of the macrophages for only a short time and are then liberated. This secondary liberation into the blood of the calcium and phosphate may be responsible for the tendency described by McLean and Hinrichs for a subsequent, delayed rise in the serum calcium described particularly after the intravenous administration of phosphate. The granules of calcium phosphate in the cells may be converted to the constituent calcium and phosphate ions which leave the cells as rapidly as formed. This process continues as the calcium and phosphate ion concentrations in the blood plasma tend to be reduced by their passage from the capillaries into the urine, feces and tissue fluid.

The sequence of events following the injection of calcium or phosphate is clearer after the intravenous administration of preformed colloidal calcium phosphate suspended in horse serum. A comparison of the blood analyses presented in table 3 of the preceding paper and table 2 in this report shows that the degree of phagocytosis of the colloidal compound is greatest shortly after the injection when the total blood calcium is greatest, and that as the plasma calcium falls, the degree of phagocytosis is gradually reduced. It is probable that there is no one point of time which separates these two phases. Rather it appears that the two overlap for a longer or shorter period of time, phagocytosis of smaller amounts of colloidal particles taking place in some cells while others are liberating their calcium and phosphate.

Two further observations may be gleaned from table 2: (a) The amount of colloidal phosphate phagocytized after 15 minutes is greater than directly after the injection. (b) The degree of phagocytosis appears to be greater first in the liver, then later in the spleen; conversely, the Kupffer cells surrender their ingested calcium phosphate more rapidly than the

macrophages in the spleen. These phenomena are probably to be correlated with the greater blood flow through the liver. This assures first a greater number of collisions between phagocyte and particle, and then later a more rapid method of "leaching out" of the ingested calcium and phosphate.

After an intravenous injection of calcium, phosphate, or colloidal calcium phosphate, phagocytosis of the colloidal compound is never observed in the lymph node. The ingestion of the colloidal particles could not be detected in the macrophages of the lymph nodes, even when as much as 100 cc. per kilo of the preformed colloidal suspension was injected intravenously over a period of six hours with the renal pedicle ligatured. Repeated intravenous injections of massive amounts of calcium and phosphate administered successively in the course of one hour or more also failed to induce phagocytosis in lymph nodes. This may be interpreted in the light of the low calcium and phosphate ion concentrations in the tissue fluid. This tissue fluid must be altered or replaced by new tissue fluid in order that the concentration of calcium and phosphate ions be raised sufficiently high to result in the formation of the colloidal compound locally in the tissue spaces. Whatever particles escape phagocytosis in the connective tissue must then find their way into the lymph node via the lymphatics to be engulfed by the macrophages. Theoretically this series of events should be obtainable. Actually, it appears that the animals die of cardiac or respiratory failure before the effective concentrations of calcium and phosphate ions can be attained in the tissue fluid.

The conditions for the phagocytosis of colloidal calcium phosphate in the lymph node may, however, be effectively produced by subcutaneous and intramuscular injections. When these are supplemented by intravenous injections of the other salt (calcium by phosphate, phosphate by calcium), phagocytosis in the lymph node is enhanced. This increase in the degree of phagocytosis may be understood only in terms of the passage of the ions injected intravenously through the capillaries and thence to the tissue fluids. This series of observations lends support to the explanation hazarded in the previous paragraph for the refractoriness of the phagocytes of the lymph node toward colloidal calcium phosphate even when it is present in the circulating blood in massive amounts.

SUMMARY

1. When colloidal calcium phosphate is caused to exist in the circulating blood, it is phagocytized by the macrophages of the liver and spleen.
2. The retention of the ingested colloidal particles by the macrophages is transient.
3. The course of phagocytosis and retention of colloidal calcium phosphate is correlated with the state of calcium in the blood.
4. Under certain conditions the macrophages of lymph nodes may also ingest colloidal calcium phosphate.

ELECTROLYTE CHANGES IN CAT MUSCLE DURING STIMULATION

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In a previous paper we have reported a loss of potassium from rat muscles as a result of stimulation (Fenn and Cobb, 1936). Similar preliminary results on cats have also been published (Fenn, 1936a). Evidence was presented to show that the loss of potassium is accompanied by a gain of sodium in approximately equivalent amount, a gain of sodium chloride, presumably in the interstitial spaces, and a gain of water, partly cellular and partly extracellular. These changes were to a large extent reversed in recovery. Some partially similar experiments on rabbits have been reported independently by Malorny and Netter (1936) showing that the gain in sodium is greater than the gain in chloride, thus confirming our experiments. Other earlier reports pertinent to this subject have been referred to previously (cf. also Fenn, 1936b). In this paper we wish to give a full report of our analyses of cat muscles, including analyses for calcium, magnesium, and phosphorus in both cats and rats. The extension of these investigations to the larger cat muscles was important because it permitted a determination of all the electrolytes on the same pair of muscles, and showed that the phenomena observed in rats are of general occurrence in other animals.

METHODS. The general methods used for analysis have been described in our earlier paper. Some details of the potassium method may be added. The Shohl and Bennett method was used after dry ashing the muscle in a muffle furnace at 500°C. in platinum crucibles. We have found no loss from known potassium samples in this process nor any loss from muscle whether all the salts were converted to sulfates previously or not. We have compared the results with those obtained by a wet ashing method on duplicate muscle samples and have detected no significant differences. Even in the latter method, the material must be heated dry before analysis in order to remove the last traces of ammonia which detracts somewhat from its value as a control. After ashing, the material is dissolved in hot, concentrated hydrochloric acid; the solution is rinsed into 15-cc. centrifuge tubes and evaporated to dryness on a steam bath in a current of air. With large samples, the solution of salts in hydrochloric acid is made up to volume in a volumetric flask and suitable aliquots containing 0.3 to 1.0 mgm. of K are transferred to centrifuge tubes and similarly evaporated to dryness. Duplicate aliquots were taken for each muscle

sample. After precipitation of the potassium chloroplatinate in these same tubes and centrifuging, the supernatant solution is removed by suction through a very fine capillary with upturned tip which is held in a stand and lowered into the tube by a screw. The precipitate is washed by the same method. After washing twice in absolute alcohol, it was found important to remove the last traces of alcohol by evaporating to dryness on a water bath. If any alcohol remains, considerably lower titration values are obtained. Further washing with strong potassium chloride, as recommended by Shohl and Bennett, was then found to be unnecessary. The original muscle samples usually weighed 1 or 2 grams and contained 3 to 6 mgm. of potassium. To facilitate drying, they were usually minced quickly in the weighing bottle with scissors.

Dry ashing was found suitable for calcium, magnesium, and phosphorus analyses. Total phosphorus was also determined in some experiments after wet ashing with sulfuric acid and hydrogen peroxide with apparently identical results (refer to table 5). Any insoluble calcium phosphate in the ash must therefore, have been minimal in amount. Calcium was precipitated with oxalate and analyzed by the manometric method (Peters and Van Slyke, 1932). In washing the precipitate, the improvements suggested by Wang (1935) were used. The magnesium remaining in the solution was precipitated as phosphate and determined by the method of Fiske and Subbarow (1929). The same method was used in analyzing for total phosphorus.

Cats were decerebrated under ether anesthesia or anesthetized with Dial (diallyl-barbituric acid plus urethane, Ciba Co.). The sciatic nerve was cut as far centrally as possible, the branch to the hamstring muscles was severed, and the nerve was drawn into a glass tube containing circular silver wire electrodes for stimulation. The knee was fixed with screw pins in a strong U-shaped clamp, and the Achilles tendon was fastened to an isometric lever for a record of the contractions.

RESULTS. *Resting muscle.* The average electrolyte composition of cat muscle in millimols (or cubic centimeter water) per 100 grams dry weight is given in table 1 (first column), together with the number of determinations which were averaged (in parentheses) and the probable errors. The table also shows similar values for plasma taken from Baumann and Kurland (1926). These may be compared with muscle when both are calculated in millimols per 100 grams of water. Omitting protein as largely inactive osmotically, the osmolar concentration is found to be higher in plasma than in muscle, the total millimols per 100 grams of water for plasma, whole muscle and fibers being 34.3, 30.2, and 29.6, respectively. This osmolar deficit in the muscle would be increased if allowance were made for the fact that some of the P belongs to the triphosphate, and would be increased further if some bound potassium were postulated. The deficit might be explained however by postulating a small amount of bound water and it would be diminished if other known substances are included. Thus if fiber proteins behave like serum proteins they might account for about 0.4 m. mols per cent. Other substances which have been omitted, such as urea and creatine, would be equal in both solutions. Carnosine, however, would raise the osmolar concentration of muscle not more

than 1.9 millimol per cent (Hunter, 1924), the amount being very variable. Anserine should also be present. (Wolff and Wilson, 1932; Parschin, 1937.)

The extracellular water has been calculated as usual from the chloride, assuming that its concentration is 1/0.97 times as great in the extracellular water with 1 per cent protein as in the plasma water with 6 per cent protein. In like manner, the cations are assumed to be 0.97 times as concentrated in the extracellular water as in plasma water. Subtracting the millimols so calculated as present in the 10.2 cc. of extracellular water (chloride space 10.2 per cent) from the total in 100 grams of fresh muscle, the amounts presumably present in the fibers are found and the corresponding concentrations are given in the last column. This subdivision of the muscle into a chloride space and a potassium space, is of course more or less arbitrary and subject to revision.

Total cation and anion equivalents are calculated after making certain assumptions concerning phosphate compounds and proteins (see legend, table 1). In whole muscle, fibers, and plasma, there is uniformly a deficit of about 2 millimols of anions not accounted for. A similar deficit was found in frog muscle (Fenn, 1936b). This must be due to an underestimation of the protein equivalents or to some other organic acids, as amino acids. The sum of the cation equivalents in muscle is 21.3 millimols, which compares well with an average value of 21.1 millimols per 100 grams obtained (by J.F.M.) by direct analysis. The larger number of total base equivalents in muscle as compared to plasma is chiefly due to the larger amount of bivalent phosphorus in muscle in place of monovalent chloride for the same osmotic pressure. This also does not indicate, therefore, the presence of bound potassium, nor indeed its absence.

The bicarbonate concentrations in the water of the plasma and fibers have been estimated at 2.2 and 1.4 millimols per 100 grams water, respectively. Taking $pK_1 = 6.1$ (horse plasma at 38°C.—Hastings, Sendroy, and Van Slyke, 1928) and pCO_2 for venous blood as 50 mm. Hg, and the solubility of H_2CO_3 as 55.4 volumes per cent per atmosphere, the pH may be calculated at 7.23 in the plasma and 7.03 in the muscle fibers. This is then additional evidence (cf. Fenn and Maurer, 1935) for a relative acidity inside the muscle fibers.

With the exception of potassium and water, the figures for muscle in table 1 apply to the gastrocnemius group of muscles which were minced, mixed together, and sampled. The potassium and water figures include analyses of the tibialis and extensor longus digitorum muscles. Examination of the data, however, discloses no significant differences in the composition of the various muscles included in these averages.

With the exception of the experiments in tables 2 and 3 all the values for potassium and water refer to muscles from which the external perimysium had been carefully trimmed off with scissors. That this makes an appre-

ciable difference in a small muscle was shown by analyzing separately, the muscle fibers (trimmed muscle) and the connective tissue (trimmings or external perimysium plus an equal or even greater weight of adhering

TABLE 1
Electrolyte balance in cat muscle

	MUSCLE			PLASMA		CHLO- RIDE SPACE	FIBERS
	mM/100 gm. dry	mM/100 gm. wet	mM/100 gm. H ₂ O	mM/ 100 gm.	mM/ 100 gm. H ₂ O	mM. in 10.2 cc.	mM/ 100 gm. H ₂ O
K (46).....	45.6 ±1.7	11.35	15.1	0.486	0.532	0.053	17.4
Na (11).....	8.6 ±0.93	2.14	2.85	16.3	17.85	1.77	0.57
Mg (6).....	4.6 ±0.11	1.16	1.54	0.099	0.108	0.011	1.77
Ca (6).....	0.35 ±0.04	0.087	0.12	0.255	0.279	0.028	0.09
Cl (17).....	5.43 ±0.46	1.35	1.80	11.7	12.8	1.35	0
HCO ₃ (5).....	4.6 ±0.3	1.14	1.51	2.01	2.2	0.23	1.40
Acid-sol. P.....	21.1	5.26	7.00	0.209	0.229	0.024	8.07
Lactate.....	1.0	0.25	0.33	0.301	0.33	0.035	0.32
Protein.....	10.8	2.6	3.46	1.10	1.20	0.02	3.97
H ₂ O, ml. (65).....	302	75.1		91.3			
Sum, millimols*.....			30.2		34.3		29.6
Sum of cation eq.....			21.3		19.2		21.7
Sum of anion eq.....			18.7		16.9		19.0

Plasma figures are from Baumann and Kurland (1926). Lactic acid and acid-soluble phosphates figures were taken from Sacks and Sacks (1933) who also gave the various phosphorus fractions as averaging (7 muscles) 20 mgm. per cent phosphorus as orthophosphate, 76 mgm. per cent as phosphocreatine, 51 mgm. per cent as adenosinetriphosphate, other fractions 16 mgm. per cent. The numbers of cations combining with each of them at pH 7.0 are 1.64, 2.0, 1.05, and 1.9 per atom of phosphorus, respectively (Hill and Kupalov, 1930). Thus an average valence of 1.65 is calculated and has been used in plasma also. The bicarbonate content of cat muscle is an average of five determinations in which the muscle sample was quickly removed and plunged into carbon dioxide-free sodium hydroxide under oil. This was acidified and the carbon dioxide liberated was caught in barium hydroxide and titrated. The plasma bicarbonate is assumed to be 45 volumes per cent for venous blood. Lactic acid concentration is assumed to be the same in the extracellular fluid as in the muscle water. For purposes of tabulation the proteins of both muscle and plasma are assumed to be monovalent, the basic equivalents for muscle protein being taken from Weber (1934) for pH 6.3 to 7.2 and for plasma from Van Slyke (1926, p. 32). The extracellular fluid is assumed to contain one-sixth as much protein as plasma, leaving 1.0 millimol difference between extracellular fluid and plasma. The distribution ratio $\sqrt{15.7/(15.7+1.0)} = 0.97$, and this figure has been employed in calculating the concentrations of anions and cations in extracellular water from their concentrations in plasma water. Of the 75.1 cc. H₂O in 100 grams of wet muscle, 10.2 cc. is assigned to the chloride space and 64.9 cc. to the fibers. The probable errors refer to the individual determinations of the series not to the means.

* Omitted proteins.

muscle fibers). Compared to the pure muscle fibers, all the connective tissue samples contained (per 100 grams of dry weight) less H_2O (av. of 4 pairs, 28 cc.) less K (av. of 4 pairs, 6.3 m. mols) and more chloride (av. of 2 pairs, 0.8 m. mols). The smaller water content of the connective tissue may perhaps be due to the more rapid drying of the small thin pieces which were trimmed off, although this was avoided as much as possible. Since connective tissue contains more chloride and less potassium than muscle, without containing more water, it seems evident that the increased chloride cannot be extracellular fluid, but must be in part inside the cells or fibers of the connective tissue. A similar conclusion was reached by Manery (1937), and by Keller and Klepetar (1934) for liver connective tissue. This characteristic of connective tissue doubtless explains why the small muscles of the foreleg, the palmaris longus and the flexor profundus digitorum, contain only 41.8 millimols of potassium per 100 grams of dry weight, while the larger biceps brachii and the triceps contain 46.7 and 47.2 millimols, respectively (averages of six muscles for each figure). No significant differences in water content of these various muscles could be detected.

Stimulated muscle. Analyses of stimulated and resting muscles from nine cats are reported in table 2, these experiments being the most complete in our series. A large number of other unpublished experiments show a similar loss of potassium and gain of water, but other elements were not simultaneously determined. The figures in table 2 confirm in all essential details, the results previously found in rats, a summary of which is included in the table for comparison. In the cats, the gain in sodium exceeds the loss of potassium by 3.5 millimols per 100 grams dry weight, and this excess is nearly equal to the gain in chloride which amounted to 4.2 millimols. Thus some of the sodium exchanged molecule for molecule with the potassium from the inside of the fibers, while the remainder came into the extracellular spaces as sodium chloride. Furthermore, if the chloride content of extra-cellular water is $12.8/0.97 = 13.2$ millimols per 100 grams water (see table 1) then the 4.2 millimols of chloride gained by the stimulated muscle may be assumed to have been dissolved in $100 \times 4.2/13.2 = 32$ cc. of water. Since the total water gained was 75 cc., the fibers must be assumed to have taken up 43 cc. due to the appearance of new solutes, thus diluting the electrolytes in the fiber water. While it seems likely that the increased amount of chloride found in stimulated muscle is due to extracellular water, there remains the possibility that the amount due to increased blood may be appreciable, even though the animals were usually bled to death and the muscle samples were always blotted firmly to remove all blood which could be squeezed out of the cut vessels.

Calcium, phosphorus and magnesium in cats and rats. Analyses for these

elements in *cat* muscles before and after stimulation are shown in table 3. The corresponding changes of other electrolytes in the same muscles are found in table 2. None of the differences in calcium or magnesium can be regarded as significant. The slight loss in phosphorus is probably real, four out of the six muscles showing a distinct loss and the other two showing no change. The loss is the more likely to be real because it is in part balanced by a gain in phosphorus which must accompany the increased amount of extracellular fluid. This must contain some phosphorus since

TABLE 2
Electrolyte changes during stimulation in cats

EXPERIMENT NUMBER	WATER		POTASSIUM		SODIUM		CHLORIDE	
	Rest.	Stim.	Rest.	Stim.	Rest.	Stim.	Rest.	Stim.
	cc.	cc.	mM	mM	mM	mM	mM	mM
1	291	384	37.8	34.3*	10.8	18.5	6.0	7.5*
2	291	345	41.5	34.7*	8.1	16.2	5.2	9.3*
3	297	387	41.9	38.6*	8.6	17.3	5.7	10.9*
4	297	382	44.0	42.2*	7.1	14.7*	4.7	11.0*
5	288	338	41.5	34.6*	9.2	14.1*	5.3	8.1*
6	318	401	47.6	40.8*	11.2	20.4*	6.5	9.6*
7	298	372	44.6	40.6*	8.4	15.1	5.5	9.0*
8	331	408	47.9	38.6*	8.3	24.8	6.4	12.7
9	279	352	41.1	39.6*	7.2	14.0	4.2	9.6
Average.....	299	374	43.1	38.2	8.8	17.2	5.5	9.7
Av. change....	+75		-4.9		+8.4		+4.2	
Av. in rats....	+49		-6.1		+8.3		+2.8	

The first of these animals was anesthetized with urethane and stimulated with a series of condenser charges for 30 minutes. All the others were anesthetized with Dial and stimulated with short tetani from a Harvard induction coil repeated every second. The duration of stimulation was 43 minutes in no. 2, 35 minutes in no. 8, 60 minutes in no. 9, and 30 minutes in all others. Averages of two or three analyses on both the resting and stimulated muscles are indicated by an asterisk. All figures are calculated for 100 grams of dried muscle.

it is an ultrafiltrate of plasma. The magnitude of this extracellular phosphorus may be estimated at about 0.09 millimol per 100 grams dry weight of muscle. The loss of phosphorus from the muscle fibers themselves must be greater than that noted in the tables by this amount. In any event, the loss is much less than might have been expected from the large increase in inorganic phosphorus which presumably occurs inside the fibers.

The slight loss of phosphorus due to stimulation was confirmed in analyses of 44 pairs of muscles from 9 different rats. One muscle of each pair had been stimulated at frequencies from 91 to 980 per minute, usually

for 30 minutes as described in our previous report (Fenn and Cobb). The stimulated muscles in all the experiments showed typical losses of K and gains in H_2O . Of these muscles, 32 pairs were wet ashed and 12 were dry ashed. The results were lower by only 0.15 m. mol. per cent dry, or 0.5 per cent by the dry ash method, showing that after dry ashing very little, if any P or Ca was left behind in the crucible as insoluble calcium phosphate. The averages of all 44 pairs of analyses in millimols per 100 grams dry were 31.1 m. mols resting and 30.7 m. mols stimulated, an average loss of 0.4 ± 0.11 m. mols (p.e. of mean). It may be concluded that in the majority of experiments a small (unimportant) loss of P probably occurs on stimulation.

TABLE 3

Total phosphorus, calcium, and magnesium in resting and stimulated muscles of cats
(Millimols per 100 grams dry weight)

EXPERIMENT NUMBER	PHOSPHORUS		CALCIUM		MAGNESIUM	
	Rest.	Stim.	Rest.	Stim.	Rest.	Stim.
4	29.2	29.2	0.29	0.34	4.58	4.80
5	28.8	28.8	0.35	0.33	4.37	4.34
6	31.6	30.9	0.38	0.37	4.72	4.53
7	29.9	29.1	0.36	0.41	4.65	4.71
8	31.8	30.8	0.44	0.47	4.85	4.33
9	28.7	28.5	0.28	0.20	4.68	4.62
Average....	30.0	29.6	0.35	0.35	4.64	4.56
Difference..		-0.4		0		-0.08

The cats were anesthetized with Dial. One sciatic was stimulated, usually for 30 minutes, with short tetani at 1 second intervals. Gastrocnemius muscles were used for analyses. The experiments are numbers 4 to 9 of table 3.

The conclusion that stimulation causes no change in the calcium or magnesium contents of muscles is further supported by experiments on 8 rats in which 19 pairs of muscles were analyzed for Mg and 17 pairs for Ca. Both resting and stimulated muscles gave an average value for calcium of 0.61 m. mol per 100 grams dry weight the probable error of the mean of the differences being ± 0.08 m. mol.

The average magnesium content was 4.89 m. mols resting and 4.90 stimulated (per 100 grams dry) and the probable error of the mean of the differences was ± 0.12 m. mol. In both the Mg and the Ca series the probable error was rather large but within these limits there is no indication of any change in either Ca or Mg during stimulation.

Total base. In the last five experiments listed in table 2, samples were taken, weighing 0.5 to 2.0 grams, usually from both the tibialis group of

muscles and the gastrocnemius group; they were analyzed (by J. F. M.) in duplicate, first for water and then, in the dried sample, for total base. The latter was determined by the method of Wright and Allison (1933) with minor modifications after dry ashing or ashing with sulfuric and perchloric acids. The method is laborious and time-consuming, but quite accurate as here used. In twenty-seven determinations of known amounts of mixtures of sodium, potassium, magnesium, and calcium, there was an average recovery of 97 per cent ± 2.8 (p.e.). In fourteen duplicate determinations on muscle samples, the average difference between duplicates was 3.7 per cent. The average total base equivalents per 100 grams of muscle water was 21.1 in cats and 20.9 in rats.

TABLE 4
Increase in total base due to stimulation
(Per 100 grams dry weight)

EXPERIMENT NUMBER	WATER		SUM OF Ca, Mg, Na AND K AT REST	TOTAL BASE		CHANGE IN TOTAL BASE	SUM OF Ca, Mg, Na AND K CHANGES
	Rest.	Stim.		Rest.	Stim.		
	cc.	cc.	m.-eq.	m.-eq.	m.-eq.	m.-eq.	m.-eq.
5	287	314	60.1	59.5	59.8	+0.3	-2.1
6	308	400	69.0	66.6	68.2	+1.6	+1.8
7g	297	370	63.0	59.5	62.8	+3.3	+2.9
7t	298	368*		62.0	63.5*	+1.5	
8g	311*	407	66.8	62.8*	71.9	+9.1	+6.8
8t	294	380		61.3*	72.3*	+11.0	
9g	272	347	58.2	58.2	74.2	+16.0	+5.0
9t	278	341		62.5	69.7	+7.2	

* Single determinations; all others averages of two. All analyses for total base by J. F. M. Numbers of experiments same as in table 3. Both gastrocnemius (g) and tibialis (t) muscles were used. Other independent determinations of water in other samples of these same muscles (except t.) are given in table 2 and show good agreement.

In the five experiments mentioned, eight pairs of cat muscle samples were analyzed, one muscle of each pair being stimulated, and the results are shown in table 4. In all cases, there is an increase in total base per 100 grams dry weight. This confirms the fact that the gain in sodium more than compensates for the loss in potassium. This increase in total base is largely attributable to the increase in extracellular water containing approximately isotonic sodium chloride, the only other important changes being an exchange of sodium for potassium from the cells. The sum of the potassium, sodium, magnesium, and calcium in resting muscles is given in table 4 for comparison with the total base. The agreement must be regarded as quite satisfactory when the large number of analyses involved is considered. A comparison between these total base changes and the

sum of the changes in sodium, potassium, calcium, and magnesium (as listed in tables 2 and 3) is shown in the last two columns of table 4. Except in experiment 9, the order of magnitude in the two columns is closely similar, and if total base changes are arranged in sequence according to magnitude, the sum of the individual changes falls into the same order except that the two largest are interchanged. Where the total base change is smallest, the sum of the individual changes shows a small decrease which is probably erroneous.

TABLE 5
Phospholipid and cholesterol in stimulated muscle
(Per 100 grams dry weight)

NUMBER	WATER		PHOSPHOLIPID		PERCENTAGE CHANGE	CHOLESTEROL		PERCENTAGE CHANGE
	Rest.	Stim.	Rest.	Stim.		Rest.	Stim.	
	cc.	cc.	mgm.	mgm.	per cent	mgm.	mgm.	per cent
6	303	400	2.90	3.30	+13.8	0.226	0.250	+10.6
7g	298	374	2.41	2.87	+19.1	0.199	0.213	+7.1
7t	298	368	3.26	3.55	+8.9	0.239	0.248	+3.8
8g	317	408	2.79	3.30	+18.3	0.237	0.229	-3.4
8t	296	380	3.21	3.65	+13.7	0.253	0.240	-5.1
9g	286	359	1.85	2.34	+26.5	0.143	0.148	+3.5
9t	268	341	3.10	2.92	-5.8	0.204	0.195	-4.4
10g*	331	446	3.32	3.61	+8.7	0.310	0.371	+19.7
10s*	335	408	2.70	2.74	+1.5	0.252	0.274	+8.7
Average	304	387	2.84	3.14	+11.6±2.4	0.229	0.241	+4.5±1.9
Expected from plasma filtration					+2.2			+21
Average of 4 rats†			4.97	5.41	+8.9	0.60	0.65	+8.3

* Rat muscles, gastrocnemius and tibialis for no. 10g; semimembranosus and biceps femoris for no. 10s. The loss of potassium in these muscles in millimols per 100 grams dry weight was 9.8 and 12.8, respectively.

† Data of Buchwald and Cori (1931).

g and t refer to gastrocnemius and tibialis muscles, respectively.

The increase in total base shown in table 4 was confirmed in two rats which gave, respectively, average increases of 1.5 and 4.5 m.-eq. per 100 grams dry weight.

Phospholipid and cholesterol. In the last four of the experiments listed in table 2, samples of resting and stimulated muscles were also analyzed (by W. R. B.) for phospholipid and cholesterol. These analyses may be conveniently reported here although the relation to the muscle electrolytes is not apparent. The method of analysis was that described by Bloor (1929) (see also Bloor, 1937). The results are calculated per 100 grams dry weight and are shown in table 5. One other experiment on rats is also

included. With one exception, all pairs of muscles showed a small percentage increase in phospholipid averaging 11.9 ± 2.4 , the probable error of the mean being sufficiently small to indicate that this change is significant. A similar average percentage increase in cholesterol during stimulation amounted to 4.5 ± 1.9 . Here three out of the nine pairs of muscles showed a loss and the probable error would suggest that the change is not significant.

These results should be interpreted, however, with reference to the changes in phospholipid and cholesterol which would be expected if the phospholipid and cholesterol in the plasma penetrated the muscle along with the chloride as a filtrate from plasma. The average gain in extracellular water of these muscles has been estimated at 32 cc. per 100 grams dry weight. Assuming concentrations of 200 mgm. per cent for phospholipid and 150 mgm. per cent for cholesterol, it may be calculated that 100 grams of dry muscle should gain 63 mgm. of phospholipid and 48 mgm. of cholesterol, which would mean an expected increase of 2.2 per cent in phospholipid content and of 21 per cent in cholesterol content. A comparison of these expected changes with the actual changes in table 5 shows that the phospholipid increased slightly more than was expected, whereas the cholesterol increase was absent or at least much less than that expected. It may be concluded from this that the cholesterol does not enter the muscle along with the plasma filtrate and behaves in this respect like the plasma proteins and calcium (Keys and Adelson, 1936). Since phospholipid, like cholesterol, is lower in lymph than it is in plasma, it is probable that this substance is also left behind in the blood to some extent during exercise, and the increase in muscle phospholipid noted in table 5 must apparently be attributed to a metabolic change, or to an intake of phospholipid by the muscle fibers from the blood plasma. Had the phospholipid in the blood been considerably higher than 200 mgm. per cent, however, the predicted increase in the muscle would be proportionally greater and the observed increase might possibly all be explained by plasma filtration, the probable error being large. An actual increase is therefore not quite proven but strongly suggested by these figures.

In some emaciated frogs, Buchwald and Cori (1931) found a 13 per cent decrease in phospholipid and a 5 per cent increase in cholesterol as a result of stimulation but reported no significant change in either of these substances in four rats after similar prolonged stimulation. Unfortunately, only in the frogs was the result calculated in terms of dry weight. Fenn and Cobb (1936) found always in rats a diminished dry weight after stimulation, the average change being from 24.4 to 21.8 per cent solids. Recalculating the results of Buchwald and Cori on this basis, the figures show an 8.9 per cent average increase of phospholipid (3 per cent decrease in one of the four rats) and an average increase of 8.3 per cent in cholesterol,

thus confirming to some extent the figures of table 5. Using the change in water contents for rats indicated in experiment 10, table 5, all of the rats of Buchwald and Cori show an increase in phospholipid.

Electrolyte changes during recovery. In rat muscles we have previously presented evidence that the typical electrolyte changes in stimulation are largely reversed during recovery. This did not occur in all the muscles of the rat as clearly as in the gastrocnemius muscle, but it was argued that a positive finding was of more significance than negative ones which indicated merely that not all the muscles of the rat recovered equally well. In a preliminary publication of a few experiments on cats, some indications of a similar recovery in this animal were reported (Fenn, 1936a). More positive data were obviously needed.

In our first 5 recovery experiments both legs were stimulated together for 30 minutes, one of them being sampled for analysis immediately afterwards, while the other was sampled after a further recovery period of 2 or more hours. In sampling, the vessels supplying the leg were first tied off.

These experiments offered certain disadvantages: 1. Any deleterious effect upon the cat resulting from sampling the first leg would inhibit recovery of the second leg. 2. There was no certainty that the loss of K during stimulation was of typical magnitude and the type of stimulation which was used, consisting of a 25 second tetanus every minute for 30 minutes does not give in general, as large a loss of K as a short tetanus repeated every 1 or 2 seconds. 3. The animals were anesthetized with dial, and we have the impression that decerebrate animals recover better. For these reasons, these experiments did not indicate as much recovery as our later work and they will not be reported in detail. It may be stated, however, that of the 14 pairs of muscles, 10 showed a gain of K during recovery; 3 showed no change and 1 showed a negligible loss. All showed (per 100 grams dry) a loss of water (av. 37 cc.) and a loss of chloride (av. 1.6 m. mols). The average gain of K was 1.5 m. mol per 100 grams dry. Two pairs of muscles were analyzed for Na and showed losses of 1.6 and 1.2 m. mols per 100 grams dry as expected.

In later experiments we used decerebrate animals and stimulated in a way which had been shown to give a sufficiently large loss of K. Moreover, the procedure was changed by stimulating one leg alone for 30 minutes and then leaving it to recover. Thirty minutes before the end of the recovery period the other leg was similarly stimulated after which the cat was bled to death and both legs were sampled together. In a control experiment we showed that, if anything, a greater loss of K resulted from stimulation early in the experiment than from a similar one several hours later. Any difference of this sort would therefore tend to diminish rather than to increase the amount of apparent recovery.

Three experiments of this type are reported in table 6. Twelve different pairs of muscles were analyzed in all, one muscle of each pair before recovery and the other afterwards. In every case, a gain of potassium and loss of water were observed. In one of these experiments, the extensor longus digitorum muscle on one side was removed before, and its mate just after stimulation, and the analyses served to show that a typical loss of potassium did actually occur in this experiment during stimulation. The average gain of potassium during recovery in these experiments was just

TABLE 6
Electrolyte changes during recovery in decerebrate cats
(Per 100 grams dry weight)

EXPERIMENT NUMBER	HOURS OF RECOVERY	MUSCLE	H ₂ O	K
	<i>hours</i>		<i>cc.</i>	<i>mM</i>
1	2.8	gm	-66	+9.7
		gl	-67	+4.4
		pl	-77	+3.3
		eld	-67	+4.0
		t	-71	+3.3
2	2.5	gm	-47	+3.6
		gl	-62	+5.5
		pl	-50	+13.1
		t	-34	+5.8
3	1.5	gm	-24	+2.2
		gl	-33	+2.5
	0.5	t	-48	+1.1
Average change in recovery.....			-54	+4.9
Change due to stimulation (exp. 2)*.....			+68	-10.3
Av. change due to stimulation (table 3).....			+75	-4.9

* Extensor longus digitorum sampled on both sides immediately after first side was stimulated.

The muscles used were gastrocnemius medialis (gm) and lateralis (gl), plantaris (pl), extensor longus digitorum (eld) and tibialis (t).

equal, as it happened, to the average loss during stimulation in the nine experiments shown in table 2. In experiment 3 (table 6) a slight variation in the technique was used, the sequence of events being as follows: 1, right leg stimulated for 30 minutes; 2, left leg stimulated for 30 minutes; 3, left leg tied off and sampled; 4, right leg sampled serially, the tibialis muscle after $\frac{1}{2}$ hour of recovery and other muscles after $1\frac{1}{2}$ hours. Recovery was found greater after $1\frac{1}{2}$ hours than after $\frac{1}{2}$ hour, as the figures show.

DISCUSSION. The data from cats presented in this paper confirm results

previously obtained with rats and little further discussion is needed. In addition, full details of our analyses for calcium, magnesium, and total phosphorus are now presented and show that these elements are not altered in concentration during stimulation (except perhaps phosphorus). We are still unable to offer any better explanations for these changes accompanying stimulation and recovery than those previously suggested. It seems most likely, however, that during activity there is a shrinking of the muscle fiber itself, the outer layers giving up their potassium in exchange for sodium. In recovery, the fiber "grows" again to its normal size with intake of K plus some anion and a corresponding loss of extracellular fluid. Since the Na never really penetrates the fiber membrane at all there is no difficulty in explaining the loss of Na during recovery.

Malorny and Netter (1936) have stressed the fact that in stimulated muscles, the gain in sodium exceeds the gain in chloride. We have shown that the amount of this excess depends simply upon the magnitude of the potassium loss. Any explanation of the one effect must include also the other. The theory offered by Malorny and Netter applied only to the gain of sodium and neglected the loss of potassium so that it is untenable in its present form.

SUMMARY

After stimulating one leg of a cat for 30 minutes, muscles of both legs are analyzed for potassium, sodium, calcium, magnesium, total phosphorus, total base, phospholipid, cholesterol, and water. The results, calculated per 100 grams of dry weight of muscle, show: 1, a loss of potassium which is balanced by a gain of sodium; 2, a further gain of sodium which is balanced by a gain of chloride and which is approximately equal to 3, the gain in total base; 4, a gain of water which is greater than the gain in isotonic sodium chloride solution; 5, no significant change in calcium or magnesium; 6, a probable slight loss in total phosphorus; 7, a slight increase in phospholipid which is greater than would be expected from the infiltration of plasma containing phospholipid, and 8, no change in cholesterol in spite of a large increase expected from infiltration of plasma, indicating that cholesterol is left behind in the blood vessels.

After a recovery period of a few hours, there is a more or less complete reversal of the changes in electrolytes which occurred during stimulation.

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FRIGHT AND DRUG CONTRACTIONS IN DENERVATED FACIAL AND OCULAR MUSCLES OF MONKEYS¹

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Pseudomotor contractures were first recognized by Philippeaux and Vulpian (1863). A review of the work bearing on contractions in denervated muscles was published by Gasser (1930). Various explanations were offered, but in 1930 Dale and Gaddum found that the pseudomotor contraction was due to activation of vasodilator nerve fibers supplying the denervated muscles. They, among others, suggested that stimulation of these vasodilator nerve fibers caused a liberation of acetylcholine at the nerve endings; the liberated substance diffused to the denervated muscle fibers and, owing to their sensitization to acetylcholine, the muscles contracted.

The present report deals with slow, long-lasting contractions in denervated muscles of monkeys occurring in the absence of electrical or direct nerve stimulation. The contractions were induced by frightening monkeys which were allowed to run loose in their cages. Contractions similar to those induced by fright were reproduced by intramuscular injection of eserine and acetylcholine, or by rapid intravenous injections of acetylcholine, with or without previous eserization.

METHODS. Monkeys (*Macaca mulatta*) were used for the majority of experiments. The weight of the animals varied between 2 and 5 kgm. The muscles denervated were those supplied by the facial, oculomotor, and trochlear nerves. All operations were carried out with aseptic technique, under sodium amytal or nembutal anesthesia administered intraperitoneally.

Observations. About two weeks after denervation (shortest period six days—longest period three weeks), contraction of the paralytic muscles occurred whenever the monkey was angered or frightened. The contractions appeared 2 to 3 seconds after the fright, increased in intensity for 5 to 7 seconds, lasted 20 seconds, and disappeared slowly within the next 15 seconds. The contractions were slow, persistent, and never as

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strong as those in the paired normal muscles. The fright reaction² was obtained in every animal tested.

The contractures in the paralytic face (see fig. 1c) were manifested by 1, a pulling of the nostril to the side of the paralysis; 2, increased wrinkling about the corner of mouth and curling of lip; 3, almost complete closure of the palpebral fissure in the vertical and horizontal diameters (sphincter effect), and 4, downward squinting of the eyebrow. During the spasm, however, the monkey was able to draw the nostrils to the normal side. When the animal directed its gaze upward, the superior eyelid moved only slightly upward, indicating the presence of a spasm in the *m. orbicularis palpebrarum*. In several animals, the upper lip on the paralytic side was curled and actively raised, exposing the teeth and gums for 8 to 10

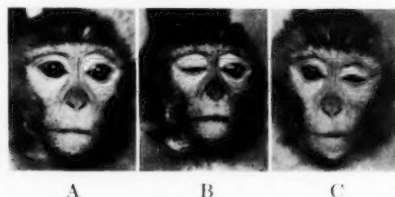


Fig. 1. A illustrates facial expression following section of the left facial nerve. Note the slight deviation of the nostrils away from the side of the paralysis and that the left palpebral fissure is slightly greater than the right.

B illustrates the absence of the blinking response in the left eyelid as part of left facial palsy.

C illustrates "the fright reaction," that is, contractions in denervated facial muscles following fright. Note the twisting of the nostrils and mouth to the left, sphincter like constriction of the left palpebral fissure and increased wrinkling of the left side of the face.

seconds. Contractions in other muscles supplied by the facial nerve could not be visualized with certainty. It was quite easy to note contraction in the muscles of the eyelids or lips because when the *m. orbicularis palpebrarum* contracts, the palpebral fissure closes, the movement of the eyelid being clearly visualized with the eyeball as a background; contractions of muscles about the lips and nostrils pull the latter from the midline and such a deviation is instantly obvious. Less prominently, contractions in the *m. depressor supercili* may be seen to pull down the eyebrow, but contractions in muscles about the ear, scalp and neck are difficult to detect in a monkey which is allowed to run loose in a cage. On close observation, however, the contractions in these muscles may be recognized by an increase in wrinkling in the overlying skin.

² For purposes of simplification, the contractions in denervated muscles following fright will be designated by the term "fright reaction."

Not infrequently, the degree of contraction in one set of muscles appeared to be greater than in the other. Sometimes the eyelids were shut almost completely while nostrils appeared in the midline; at other times, the eyelids remained open while the nostrils and upper lip twisted to the side of the paralysis.

The fright reaction in monkeys with ophthalmoplegia was manifested by a slow elevation of the drooped eyelid, the palpebral fissure remaining open for 20 seconds and gradually closing again in the next 15 seconds. (See fig. 2, A to D.) Contractions in other ocular muscles were not so conspicuous. In several animals, slight internal rectus motion was detected. In one monkey, in which the homolateral third and fourth cranial nerves were sectioned, the globe on the affected side moved downward

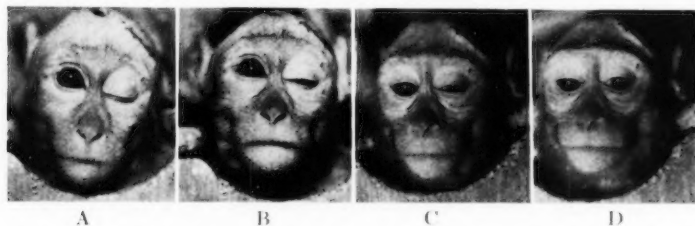


Fig. 2. A illustrates complete ptosis following section of the left oculomotor nerve. Other evidence of ophthalmoplegia may be seen in figure 2 D, that is, external deviation of eyeball, absence of vertical movements, dilated and fixed pupil.

B, C and D show various stages of elevation of ptosed eyelid. The elevation was effected by intramuscular injection of eserine followed by acetylcholine. Photographs were taken every few seconds while the acetylcholine effect was taking place. The same opening of the eyelid was elicited by frightening the animal (fright reaction).

and somewhat inward in association with fright. It is probable that the downward movement of the globe in the latter instance was the result of a spasm in the denervated superior oblique muscle. Ordinarily, vertical movements of the eyeball with denervated ocular muscles do not occur under conditions of fright nor after the introduction of eserine and acetylcholine into the body. The lack of vertical movements is the result of contractions in all muscles denervated by the ocular nerve section. Since the superior rectus, the inferior rectus and inferior oblique muscles are antagonistic in their actions, no vertical movement could occur. In an animal in which the third cranial nerve was cut and the inferior rectus muscle resected, slight upward movement of the eyeball was observed after injections of eserine and acetylcholine. This demonstrated the fact that when the pull of the inferior rectus on the globe was eliminated, contraction in the denervated superior rectus muscle became apparent.

The mode of eliciting fright reaction. The method commonly employed to frighten the animal was to threaten it with objects such as sticks, black rubber hose, or a monkey catching net. When the monkey was thus frightened, it ran around the cage dodging the menacing object. About two or three seconds after the threatening had ceased, slow contractions of the paralyzed muscles appeared.

An interesting feature was noted in the relation of the fright reaction to the type of stimulus used and also to the time the stimulus was applied to frighten the monkey. For instance, if the animal were frightened more than once or twice within a short period by one method, the contractions in denervated muscles became less conspicuous after the first fright reaction. The more attempts made to frighten the animal, the less intense became the reaction. The longer the period between the threatenings, the better was the chance to obtain a good fright reaction. Every time the animal was frightened by new methods, contractions were invariably elicited.

The reaction appeared to be more intense with sudden fright or sudden effort. For instance, when a monkey held in the examiner's hands made a sudden attempt to escape and after a very short struggle returned to its cage, contractions in denervated facial or ocular muscles appeared and these were more prominent than the contractions obtained by simple threatening.

It seemed that strong muscular effort augmented the fright reaction. Exercise alone yielded contractions in the denervated muscles of some but not all monkeys. Since the element of fear could not be eliminated in animals which were chased, it could not be determined with any degree of certainty whether muscular effort alone could produce contractions in denervated muscles. Furthermore, repeated exercise after the initial fright reaction had no visible effect on the state of denervated muscles. That fear alone could produce contraction in denervated muscle was proven by the contraction in paralyzed facial muscles in a blind and hemiplegic monkey which remained still while frightened by a jarring of its cage. The fright reaction was obtained so long as the muscles remained denervated.

Action of drugs. The contractions in denervated muscles associated with fright could be reproduced by parenteral injections of eserine and acetylcholine (fig. 2, A to D, and fig. 3). At about the same time, the fright reaction first appeared, contractions in denervated muscles could also be elicited by intravenous or intramuscular injections of acetylcholine. When evidence of nerve regeneration became manifest, the acetylcholine effect began to decrease. It disappeared entirely when nerve regeneration was complete. There was close parallelism between the contractions associated with fright and those reproduced by eserine and acetylcholine.

Eserine. In order to make intramuscular injections of acetylcholine effective, it was necessary to eserinize the animal. Eserine appears to have a specific effect in inhibiting the destruction of choline esters in the body (Engelhart and Loewi, 1930; Matthes, 1930). When circulating in the blood stream, eserine permits acetylcholine, which otherwise would be destroyed, to act. Eserine (physostigmine salicylate), injected intramuscularly in doses larger than 0.05 mgm. per kgm. of body weight, produced diffuse fibrillar twitchings of all normal skeletal muscles. These fibrillary twitchings first appeared 10 to 15 minutes after the injection of eserine and persisted for 30 to 40 minutes. The twitchings were conspicuously absent in denervated muscles. These results may be explained by the theory that eserine, inhibiting the choline esterase, prevented the destruction of excess acetylcholine formed at normal nerve endings. The excess acetylcholine caused the muscles to contract.



Fig. 3. A illustrates facial expression at rest.

B Contraction in the denervated muscles of the left face following intramuscular injection of eserine and acetylcholine. Compare with figure 1.

In the case of denervated muscles, there was no acetylcholine formed at the degenerated motor nerve endings. Therefore there were no twitchings in these muscles. Only when acetylcholine became available, either from an endogenous or exogenous source, did the denervated muscles, more sensitive to acetylcholine than normal muscles, go into a slow and lasting contracture. In order to avoid confusion between twitchings of normal muscles obtained with eserine and contractions of denervated muscles obtained with eserine and acetylcholine, the dose of eserine injected was reduced to 0.05 mgm. or less per kilogram of body weight. With such a dose of eserine, twitchings in normal muscles were not visible.

Injections of eserine potentiated contractions in denervated muscles associated with fright. In every instance, the augmentation became evident from 10 to 20 minutes after eserization. The interval between the eserization and the appearance of the intensification of the fright reaction was equal to the interval between eserization with dose larger than 0.05 mgm. and appearance of twitchings in normal muscles. The potentiation of the fright reaction by eserine suggested that the contrac-

tions in denervated muscles associated with fright were effected through acetylcholine.

Acetylcholine. Acetylcholine injected intramuscularly without previous injection of eserine had no effect on denervated muscles. It was necessary to give eserine 15 minutes before injections of acetylcholine. If the period between eserine and acetylcholine administration were less than 15 minutes, then contractions in denervated muscles were less conspicuous or absent. The shortest interval between injections of eserine and acetylcholine which would favor a good contraction was approximately equal to the time it took eserine to cause muscular twitchings in normal muscles. The usual dose of acetylcholine for intramuscular injections was 0.8 mgm. per kilogram of body weight. Within forty seconds after the injection of acetylcholine, denervated muscles went into a slow contraction which lasted from one to thirty minutes. The usual duration, however, was between 5 and 10 minutes.

Acetylcholine injected rapidly by vein, even without eserization, produced contractions in denervated muscles; the vein usually selected was the saphenous. These contractions appeared from two to three seconds after the infusion and lasted from thirty to forty seconds. The amount of acetylcholine given intravenously was 0.4 mgm. per kilogram of body weight. In some animals, a second intravenous injection, following by 1 or 2 minutes upon the first, was ineffective.

Acetylcholine given intravenously with eserization produced contractions in denervated muscles which lasted only 30 to 40 seconds. The only difference was that in the eseritized animal, the dosage was one-tenth of the dose which produced the same effect in the uneseritized animal.

Contractions in denervated muscles were not the only changes produced by acetylcholine. Consistent manifestations were salivation, chewing and grinding of teeth, slowing of the heart rate and hypoactivity. The less constant symptoms were lacrimation, defecation, urination, blushing of the skin of the face, and prostration. Occasionally, depending upon amounts of eserine injected, acetylcholine produced twitchings in normal muscles. The general effects lasted anywhere from 30 to 50 minutes. Although blood pressure readings were not recorded, it was conjectured that the hypoactivity and prostration were results of lowered blood pressure.

Atropine. The prostration, hypoactivity, increased salivation, and lacrimation were invariably prevented by previous injection of atropine sulfate, 0.4 mgm. per kilogram of body weight. The total dose was never more than 2.0 mgm.; the usual dose was 1.0 mgm. Atropine did not have any visible effect on the state of contraction in denervated or normal muscles. In all animals in which atropine was injected intramuscularly, mydriasis occurred.

Adrenaline. Adrenaline never produced contractions in denervated facial or ocular muscles. The drug was given intravenously or intramuscularly with dose ranging from 0.025 to 2.0 mgm. On several occasions, a widening of the palpebral fissure in an ophthalmoplegic eye occurred after an intramuscular injection of adrenaline. It was found, however, that the opening of the fissure took place soon after the animal was caught, even without administration of adrenaline; and when epinephrine was given, after the monkey had been quiet for a minute or two, very little, if any, opening of the fissure occurred. Evidently the raising of the eyelid observed on several occasions after injections of adrenaline was part of the "fright reaction."

Indeed, not only did epinephrine fail to produce contractions in muscles, but it actually inhibited the contractions associated with fright or by injections of acetylcholine. During the period of prolonged contraction in denervated facial muscles obtained by eserine and acetylcholine, injection of epinephrine caused a temporary lessening of the spasm in these muscles. In some animals with ocular denervation, the retraction of the upper eyelid under influence of eserine acetylcholine was diminished by adrenaline. The lessening of the retraction, however, could not be demonstrated in all instances because epinephrine causes a widening of the palpebral fissure under normal conditions. The inhibitory effect of adrenaline upon the state of contraction in denervated facial or ocular muscles was not always demonstrable.

The rôle of sympathetic innervation. From the foregoing data, it appears that contractions in denervated muscles associated with fright were effected through a substance similar to acetylcholine. The following experiments were carried out to determine whether this substance (acetylcholine) reached the muscles in question through the local vasodilator nerve supply or by diffusion from the general circulation through the capillary walls in contact with these muscles.

The nerve supply to the blood vessels of ocular muscles is derived mainly from the cervical sympathetic, while nerves of blood vessels to the face come from the cervical sympathetic and the infraorbital nerves.

Cervical sympathetic. Stimulation of the cervical sympathetic trunk produced the usual dilatation of the pupil, slight exophthalmos, and piloerection of the face. No visible contractions in denervated facial muscles were noted, even after strong faradic current was used. In the ophthalmoplegic eye electrical stimulation of the cervical sympathetic caused a slight elevation of the ptosed eyelid, but the degree of elevation was not more than that in the normal eye. The slight retraction on stimulation of the sympathetic never compared with the marked retraction of the ptosed lid associated with fright.

The results from stimulation of the cervical sympathetic were constant

at all times before and after a period of degeneration of motor nerve endings to the ocular or facial muscles. Section of the cervical sympathetic or extirpation of the superior cervical ganglion on the side of the denervation of facial or ocular muscles, or on both sides, did not prevent the occurrence of the fright reaction. Similar observations were also made by Mahoney and Sheehan (1936), who extirpated both stellate ganglia in addition to both superior cervical sympathetic ganglia and trunks.

Infraorbital nerves. Euler and Gaddum (1931) found after removal of superior cervical ganglion that contractions in the zygomatic muscle of dogs could be elicited by stimulation of the infraorbital nerve on the side of facial denervation. This result, however, could not be reproduced in monkeys. Stimulation of the nerves leaving the three infraorbital foramina produced slight movement of the upper lip, but when care was taken to prevent electrical spread to contiguous structures, no contractions occurred.

Resection of the homolateral superior cervical ganglion, cervical sympathetic, and infraorbital nerves did not in any degree reduce fright contractions in denervated facial muscles. That sympathectomy to the face was complete was illustrated by the following animal in which the motor nerve supply to the face and ipsilateral superior cervical sympathetic ganglion and trunk and infraorbital nerves were resected. Injection of eserine and acetylcholine produced a persistent contraction in denervated facial muscles. During the height of the contraction induced by acetylcholine, an intravenous injection of adrenaline produced a marked blushing on the normal side of the face, while marked blanching of the face, conspicuous dilatation of the pupil, increased lacrimation on the sympathectomized side, and a diminution of the contraction in denervated facial muscles occurred on the other side. These were sensitization effects obtained with adrenaline and were proof that the post ganglionic sympathetic fibers to the face and eye had been cut.

DISCUSSION. It has been shown that denervated ocular and facial muscles contracted in association with fright and that these contractions were augmented by eserine and reproduced by parenteral injections of acetylcholine. These contractions occurred so long as the muscles remained denervated, and strongly suggested that the fright reaction was due to secretion of acetylcholine.

Although eserine potentiated, it did not prolong the fright reaction. In keeping with this, eserine potentiated but did not prolong acetylcholine contractions of denervated muscles when the drug was given intravenously. Eserine, however, did prolong the contractions obtained by intramuscular administration of acetylcholine. *These findings indicate that acetylcholine may persist in the body tissues for long periods.* Only when acetylcholine gets into the circulation is it so rapidly destroyed.

The problem of the site of formation of acetylcholine in the body and how this substance reaches the denervated muscles under conditions of fright has not been solved. One possibility is that during fright the autonomic system is activated and, at vasodilator nerve endings, acetylcholine is formed. It was found, however, that neither stimulation nor resection of the sympathetic nerves supplying the area of the denervated muscles in question visibly altered the contractions obtained with fright or by acetylcholine. These negative findings are stressed because Rogowicz (1885) found contractions in the upper lip although not in the orbicularis palpebrarum in denervated facial muscles of a dog on stimulating the cervical sympathetic. Also Hinsey and Cutting (1933) pointed out that contractions in denervated gastrocnemius muscle of a cat could be produced by strong, rapid faradic stimulation of post-ganglionic sympathetic fibers.

It is possible, however, that the vasodilator nerve fibers to the ocular and facial muscles originate from sources other than the cervical sympathetic, e.g., the trigeminus. The latter source was partially eliminated in animals with facial denervation when the infraorbital nerves were sectioned. Certainly cervical sympathectomy and division of the infraorbital nerves should have diminished contractions in paralyzed facial muscles induced by fright to some degree, but no such lessening occurred.

If the fright reaction were not due to a local secretion of acetylcholine, then one might postulate that the "parasympathetic humoral substance" reached the denervated muscles through the circulation. The fact that Freeman and Cannon (1931) could not demonstrate acetylcholine in the blood after stimulation of the vagus, as Brinkman and Velde did in 1925, does not detract from the possibility that during fright or under conditions of strong emotion, where the entire organism is activated, an excess of acetylcholine-like substance is secreted in all parts of the body, such as at endings of autonomic and motor nerves, at sympathetic ganglia, and perhaps at many other organs, including the adrenals. A part of this excess of acetylcholine which could not be so rapidly destroyed in the body tissues (compare with deduction in last 2 lines on page 616) may have escaped into the general circulation and thus reached the sensitized denervated muscles. The discharge of a parasympathetic substance may play a rôle in parasympathetic activity such as defecation and urination which are known to occur during fright.

Irrespective of the question whether acetylcholine is formed locally, or whether it reaches the denervated muscles through the general circulation, there appears to be cogent evidence that this substance is secreted in the body during fright, the sensitized denervated muscle acting as an indicator of the presence of acetylcholine. It is suggested therefore that during fright, there is a general discharge of the autonomic system and

a secretion of adrenergic and cholinergic hormones. These hormones produce their effects partially independent of the autonomic nerve supply, acting on their specific organs. The interaction between the two hormones influences the body in such a manner as to protect it in time of emergency. The contraction of a paralyzed muscle during fright illustrates one of the parasympathetic actions in body defense mechanism.

Additional observations made on these animals revealed that humoral control of body processes was found to vary with different animals in the mammalian scale. Using the monkey, mangabey, baboon, and chimpanzee as examples, it was found that the higher the animal was in the phylogenetic scale, the less conspicuous were the contractions in the denervated muscles induced by fright or those reproduced by acetylcholine.

SUMMARY

1. Denervated facial and ocular muscles of monkeys were found to contract during fright (fright reaction).
2. These contractions were invariably reproduced by parenteral administration of acetylcholine.
3. The fright reaction was augmented by previous eserization.
4. The contractions obtained with fright and those with acetylcholine made their first appearance after a period sufficient to allow the nerve endings to degenerate. The contractions in denervated muscles with fright and those reproduced by acetylcholine could not be elicited when nerve regeneration had occurred.
5. The strict parallelism between the contractions obtained with fright and those reproduced by acetylcholine suggested that the fright reaction was due to a substance like acetylcholine which was secreted in the body during fright, the denervated muscles thus acting as an indicator of the presence of acetylcholine in the body.
6. Adrenaline did not produce contractions in denervated muscles; it, moreover, inhibited the contractions in denervated muscles produced by acetylcholine.
7. Neither stimulation nor section of the sympathetic nerve supply to the muscles in question produced any visible effects on the state of denervated muscles.
8. It is suggested that during fright there occurs a general discharge of the autonomic nervous system with a secretion not only of a sympathetic-adrenergic hormone, but also of a parasympathetic-cholinergic substance, acetylcholine, both of which enter in the rôle of body defence mechanisms in time of emergency.

I am indebted to Prof. J. F. Fulton for his invaluable assistance and counsel in this work. I am much indebted also to Dr. Robert S. Dow for

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EFFECTS OF CRYPTORCHIDISM AND CASTRATION ON EXOPHTHALMOS IN RABBITS AND GUINEA PIGS

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In previous papers (1, 2) we have pointed out that exophthalmos developed more frequently in male rabbits—60 per cent vs. 40 per cent. This is independent of the exophthalmos-promoting effect of thyroidectomy which affects both sexes alike. Recently (3) we have reported that gonadectomy in the adult male prevented the development of thyroidectomy exophthalmos and if exophthalmos was present at the time of gonadectomy it receded rapidly (2-3 weeks).

We have now made attempts to analyze the gonadal factor in exophthalmos by means of cryptorchidism. Permanent cryptorchidism was produced in thyroidectomized rabbits by the method of cutting the gubernaculum and anchoring the anterior end of the testis to the lateral abdominal wall at the level of the umbilicus by a single silk suture through the tunica albuginea. This causes degeneration and absorption of the germinal epithelium with preservation of the Sertoli cells and the interstitial cells. Recent work (Nelson, 4, Hanes and Hooker, 5, and others) suggests that the hormone production of the interstitial cells of cryptorchids is decreased despite their anatomical hypertrophy. Sufficient functional activity, however, is preserved to maintain the prostate, seminal vesicles, Cowper's glands, and the sexual potency of the animal. Typical instances of the effect of gonadectomy and cryptorchidism on exophthalmos are given in the following table.

Rapid regression of fully developed thyroidectomy exophthalmos is seen following gonadectomy in the rabbits (nos. 3 and 4), while in their controls (nos. 1 and 2) the exophthalmos remained unchanged or even slightly increased.

Cryptorchidism, on the other hand, does not cause regression of the exophthalmos even after 6 months and, in some instances, appears slightly to favor further development. It may be concluded from such experiments that the germinal epithelium has little if anything to do either with the development of or the regression of this form of exophthalmos, but that the functional activity of the remaining portion of the testis clearly prevents regression of the exophthalmos and often favors a slight increase.

What hormones of the cryptorchid testis or possibly of the tissues controlled by it (prostate, seminal vesicles, Cowper's glands) or influenced by it (anterior pituitary, suprarenal cortex) is responsible is not known.

In the guinea pig the effects of castration and cryptorchidism on exophthalmos differ somewhat from those present in the rabbit. A remarkable sex difference in the incidence of exophthalmos was observed in a batch of 23 guinea pigs received from a breeder. Eight of the 11 males on arrival at the laboratory had exophthalmos, varying from +? to + and 3

TABLE 1

RABBIT NUMBER	OPERATIVE PROCEDURES	EXOPHTHALMOS BEFORE GONADECTOMY (WEEKS)				EXOPHTHALMOS AFTER GONADECTOMY (WEEKS)						
		4	3	2	1	1	2	3	4	5	6	7
1	Thyroidectomy	2+	2+	2+	2+	2+	2+	2+	2+	2+	2+	2+
2	Thyroidectomy	+?	+?	+	+?	+	+	+	+	+	+	+
3	Thyroidectomy; gonadectomy	+	+	+	+	+?	-?	-	-	-?	-?	-
4	Thyroidectomy; gonadectomy	+?	+	+	+	+	-?	-?	-	-?	-	-
		EXOPHTHALMOS BEFORE TESTES SUSPENDED				EXOPHTHALMOS AFTER TESTES SUSPENDED						
		4	3	2	1	1	2	3	4	5	6	7
5	Thyroidectomy; testes suspended	-?	-?	-?	-?	+?	±	±	-?	+?	+?	+?
2	Thyroidectomy; testes suspended	+	+	+	+	+?	+	+	+	+?	+?	+?
6	Thyroidectomy; testes suspended	+?	+?	+?	+?	-?	-?	-?	-?	+?		
7	Thyroidectomy; testes suspended	-?	-?	-?	-?	-	-?	-?	-	-?	+?	
8	Thyroidectomy; testes suspended	-	-	-	-	-	-	-	-	-	-	

varying from - to -?, while none of the 12 females had any exophthalmos. Five males, including 4 with + exophthalmos, were castrated and 5 males, including 4 with + exophthalmos, were kept as controls. All were maintained under similar conditions as regards cages and food (whole oats, alfalfa hay, lettuce). The exophthalmos was fully maintained in the castrates at the end of 20 days when they were used for other experiments, while the exophthalmos in the intact controls completely disappeared within 8 days. This result is apparently opposite

to that found in rabbits and no explanation is at hand to account for the persistence of exophthalmos in the castrated guinea pigs or the rapid regression of exophthalmos in the intact controls, unless the partition of function between the suprarenal cortex and testis is different from that of the rabbit.

That the exophthalmos was of the ordinary type due to increased tonus of the orbital muscles of Mueller from central stimulation is shown by its instant conversion into an enophthalmos when the cervical sympathetic trunk was divided.

TABLE 2

GUINEA PIG NUM- BER	WEIGHT AT TIME TESTES SUSPENDED	EXOPHTHAL- MOS BEFORE TESTES SUSPENDED (DAYS)		EXOPHTHALMOS AFTER TESTES SUSPENDED (DAYS)																
		6	4	2	2	4	6	8	10	12	14	16	18	20	22	24	26	28	30	32
		grams																		
1	660	-	-	-?	±	±	±		±	+	+		+		+		±	±		-?
2	418	-	-	+	+	+	+		+	+		+		+	+		±	±		±
3	621	-?	-	±	+	±	±		±	+	+		+		+		±	±		-?
4	284		-	+	±		-?		+	±	+	+	+	+			±	-		±
5	273		-?	-	-?		-?		-?		-	±	+	±						
6	478		-	-	-		+	+	+	+	-?	-	+	+	±				-?	±
7	526	-		-	-?		+	±	±	±	+	+	+	+	±	+	+	+	+	+
8	552		-	-?	±	+	+	±	±	±	+	±	±	-	-	+	-			-
9	262			+	±	±	±	±	±	±	+	+	+	+						
10	322			+	±	±	±	±	±	±	+	+	+	+	±	±	+	+	+	
11	298			+	±	±	±	±	±	±		±	±	+	+		+	+	+	
12	514			±	±	+	+	-?	±	±				-?	-?		+	+	+	
13	237		±	+	+	+	+		±	±		+		+						
14	226		-	-?		±			±	-	+									
15	349		-	±		-	+	+	+	+										
16	534	-?	±	-	-		-?		-	±	-?		-	-	-					
17	526		±	-	-		-		-	±			-	-		-				
18	217		-	-	-		-		-	-	-?									
19	278		-	-?		+	+	-?	-?	-?	-?									

Cryptorchidism in guinea pigs produces a slight but transient exophthalmos.

In 14 out of 19 animals a transient exophthalmos was observed. The eyes began to be more prominent two days after suspending the testes and reached their greatest protrusion about the end of the second week on an average. At 30 days there was usually but not always some regression of the exophthalmos and in some guinea pigs it had disappeared altogether.

The effects of cryptorchidism on exophthalmos in the rabbit and guinea

pig differ mainly in degree. In rabbits it does not cause exophthalmos under our experimental conditions but will maintain an existing exophthalmos indefinitely, while in guinea pigs it causes a slight, highly variable, transient exophthalmos.

We have many times pointed out that the most marked cases of exophthalmos occurred in sexually very active male rabbits—a condition that frequently follows thyroidectomy at puberty in this species.

This stimulating effect of thyroidectomy on the gonads has been assumed to be due to the intense stimulation and hypertrophy of the anterior pituitary which follows thyroidectomy in all mammals. Thyroidectomy also appears to lessen the adrenotropic effect of anterior pituitary extracts (Loeser (6), Emery and Winter (7), McQueen-Williams (8)).

The promoting effect of thyroidectomy on exophthalmos production may be due, therefore, either to an actual increase in the activity of the interstitial cells or to the lessening of a protective or counteracting influence from the adrenal cortex. Further, we have some evidence (unpublished) that adrenal injury in the rabbit tends to eliminate the sex difference in the incidence of exophthalmos and, in addition, slightly promotes the development of exophthalmos, especially in the female. Such evidence suggests that the promoting effect of interstitial cell activity on exophthalmos may be due to a relative and not necessarily an actual increase in some hormone or hormones produced by it.

Other more general factors are involved in exophthalmos production. It has been repeatedly emphasized that diet abnormalities, particularly disturbed calcium-phosphorus ratios, are necessary. It is also significant that in all of the diseases in which exophthalmos occurs (exophthalmic goiter, experimental rickets and osteoporosis, acromegaly, etc.) there are disturbances of the mineral metabolism.

SUMMARY

In adult rabbits gonadectomy causes regression of existing exophthalmos in two to three weeks. Cryptorchidism does not cause regression of existing exophthalmos nor does it appear to produce exophthalmos. This indicates that stimulation of the central sympathetic mechanism which causes exophthalmos is promoted by some hormone produced by the interstitial cells and transmitted through the blood stream. A deficiency of thyroxine is a necessary factor in this central sympathetic stimulation.

In the guinea pig castration does not cause regression of existing exophthalmos as in the rabbit. Cryptorchidism causes a slight and transient exophthalmos in guinea pigs even with intact thyroids. These differences between the rabbit and guinea pig suggest that hormones of the adrenal cortex may also play a rôle and that the partition of gonadal function between adrenal cortex and interstitial cells differs in the rabbit and guinea pig.

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THE INTERRELATIONSHIP OF THE PITUITARY GONADOTROPIC HORMONES IN FOLLICULAR DEVELOPMENT AND OVULATION OF THE JUVENILE RABBIT

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Previous reports from this laboratory have presented data concerning the interrelationship of follicle-stimulating and luteinizing pituitary extracts in the ovulation processes of anoestrous cats (3), oestrous and hypophysectomized adult rabbits (4), (5), and immature rabbits (4).

In the cat it was found that several factors were involved in the pre-ovulatory growth of follicles. The most important finding was that follicles developed by pure follicle-stimulating hormone (F.S.H.) were more responsive to an ovulating mixture administered intravenously than were follicles stimulated by similar extracts containing relatively small amounts of luteinizing hormone (L.H.). Mild atresia such as resulted from injections of unfractionated pituitary extracts, or from prolonged treatment with F.S.H. containing small amounts of L.H. always reduced the sensitivity of the follicles to the ovulating mixture. It was also found that certain combinations of the two pituitary extracts had decided advantages over either hormone alone when used as the intravenous stimulant for ovulation. The follicle-stimulating extract alone gave poor results, but when 0.5 gram (1 r.u.) was combined with traces of L.H. uniform ovulation of as many as 30 ova per individual resulted. The addition of progressively increasing amounts of L.H. to the same amount of F.S.H. gave a rapidly decreasing number of ruptured follicles until no ovulation was found.

In general, the same factors controlling ovulation in the anoestrous cat were found to be effective in the oestrous rabbit. Hypophysectomy (5) in such animals resulted in atresia so that direct intravenous injection of gonadotropic preparations was ineffective for ovulation. However, by stimulating the medium and small follicles with F.S.H., thereby replacing the larger atretic follicles, ovulation could be induced with F.S.H. plus small amounts of L.H. injected intravenously. It was also demonstrated that L.H. alone or combined with F.S.H. was essential for corpus luteum secretion after induced ovulation under the above conditions.

In this paper we wish to present further studies on the effects of various

mixtures of the pituitary gonadotropic hormones on the development of the follicle and its ovulation in the immature rabbit.

The gonadotropic preparations used in these experiments were prepared from horse, sheep and hog pituitary powder after the methods used in this laboratory (2). One hundred fifty 12 to 14 week old chinchilla rabbits weighing 1200 to 1500 grams were used as experimental animals. Dosages employed are stated in terms of dry pituitary powder weight and in rat units (20 to 25 mgm. ovaries in three 21 day old rats).

Follicular growth. It has been generally known that intravenous injection of large doses of gonadotropic preparations into juvenile rabbits fails to elicit ovulation. However, after the follicles have been developed by suitable pituitary extracts to a size and maturity simulating those of the oestrous adult, an intravenous injection of a proper ovulating mixture almost invariably results in the rupture of unusually large numbers of

TABLE 1
Follicular growth

TREATMENT	RESULTS	
	a. Before intravenous injections	b. After intravenous injections
Sheep F.S.H. 0.5 gm. (1 r.u.) daily for 5 da.	Follicles only	40-60 ovulation points
Same F.S.H. plus trace L.H. (1-2 mgm. eq.)	Atresia and luteinization 80% ovulated spontaneously 20% did not ovulate	No further ovulation
Same F.S.H. plus more L.H.	Atresia and rapid luteinization with entrapped ova no ovulation	5-10 ovulation points No ovulation

follicles. In this study we have attempted to determine the hormonal stimuli essential for this normal follicular growth. The follicles were grown by the subcutaneous injection of purified follicle-stimulating extracts alone or in combination with different amounts of luteinizing extracts. This was followed by a single intravenous injection of a mixture of the two extracts known to be optimal for ovulation, and the number of ovulation points was recorded.

The procedure was as follows: 21 immature rabbits were injected subcutaneously with 4-10 r.u. of F.S.H. alone and mixed with varying amounts of L.H., the total amount being distributed in equal doses twice daily for five days. On the fifth day a laparotomy was performed to determine the degree of follicular enlargement. At the same time the ovulatory mixture was given intravenously.

It was found (table 1) that purified F.S.H. caused only pure follicular

growth without atresia, cystic degeneration or ovulation. The intravenous injection of an ovulating mixture in animals treated with F.S.H. subcutaneously for five days resulted in ovulation of 40 to 60 follicles with subsequent luteinization and pseudopregnancy. The follicles developed by F.S.H. were apparently in the proper physiological state to respond to the hormonal stimulus for ovulation (fig. 1).

In contrast to these, follicles which were grown with a mixture of F.S.H. plus small amounts of L.H. often ovulated spontaneously. Subsequent intravenous injection of such animals failed to produce additional ovulation, but in those which did not ovulate spontaneously a small number of follicles ruptured. The relatively small number of ruptured follicles may be explained by the fact that this combination of the two

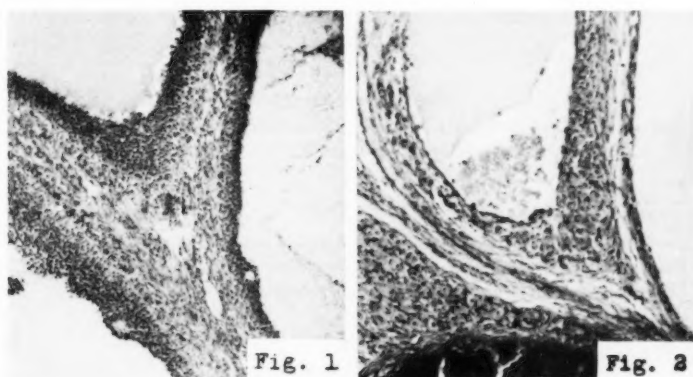


Fig. 1. The walls of graafian follicles treated with F.S.H. Note the thick layer of proliferating granulosa cells.

Fig. 2. Follicles after administration of F.S.H. plus small amount of L.H. Note the absence of granulosa cells due to cystic degeneration and luteinization.

hormones produced corpora lutea and cystic follicles, many of which showed advanced atresia (fig. 2).

When larger amounts of L.H. (3 to 15 mgm. eq.) were added to the F.S.H., and the mixture injected subcutaneously, spontaneous ovulation did not occur nor could ovulation be induced by intravenous injections of the ovulating mixture. This lack of responsiveness was associated with marked atresia and cystic degeneration of the larger follicles and premature luteinization which entrapped the ova of smaller follicles. It may also be mentioned that the uteri usually showed a plus one or plus two pseudopregnant reaction.

When the amount of L.H. was increased to 20 mgm. equivalent or more, luteinization was more rapid and atresia more marked while in-

travenous injection again consistently failed to elicit ovulation. The uteri of such animals usually showed maximal pseudopregnancy changes.

Experimental ovulation in juvenile rabbits. In order to determine the optimal conditions for inducing ovulation of follicles which had been grown experimentally, the ovaries of a series of animals were prepared by giving subcutaneous injections of 0.5 to 2 r.u. of F.S.H. daily for 5 days. Six to ten hours after the last injection a laparotomy was performed to determine the gross appearance of the ovaries. At the same time a definite amount of F.S.H., L.H. or a combination of the two hormones was given by intravenous injection. The ovaries were examined again 24 to 48 hours later and the ovulation points in each ovary counted.

Since the results obtained from sheep, horse and hog pituitary extracts differed markedly in the degree of response elicited, data were grouped according to the source of extracts employed.

TABLE 2

Sheep pituitary extracts

This entire group of animals was given a preliminary treatment of 1-2 r.u. (0.6 gm. eq.) of F.S.H. daily for 5 days subcutaneously.

NUMBER OF ANIMALS	INTRAVENOUS INJECTION	NUMBER OVULATING	TOTAL NUMBER OF OVULATION POINTS
18	0.5 gm. F.S.H. (1 r.u.) alone	10	225
11	Same F.S.H. plus 5 to 20 mgm. equivalent of L.H.	11	238
5	Same F.S.H. plus 100 to 500 mgm. equivalent of L.H.	3	12
12	0.1 to 1.0 gm. eq. L.H. alone	8	71

With the use of sheep pituitary extracts (table 2) it was found that subcutaneous administration of F.S.H. (1 r.u. daily for 5 days) resulted in a 3- to 5-fold increase in ovarian size which was due to growth of the medium and large follicles. This was accompanied by the usual hypertrophy and increased vascularity of the uterus. When subcutaneous injections of F.S.H. were discontinued, the follicles receded fairly rapidly without ovulation or luteinization intervening. However, intravenous injection of F.S.H. shortly after the last subcutaneous injection resulted in ovulation of 10 animals out of a group of 18. Luteinization followed ovulation and within a few days the ovaries contained solid lutein tissue with uteri showing maximal pseudopregnancy changes. The animals failing to ovulate on identical treatment were found to have marked follicular atresia, although the ova still contained germinal vesicles. The uteri were characteristic of the oestrous state.

Intravenous injection of a similar dosage of F.S.H. to which 5 to 25

mgm. eq. of L.H. had been added was found in all cases to be extremely effective in eliciting ovulation after preliminary follicular stimulation, and the number of ovulation points was approximately the same as obtained after F.S.H. alone. Since F.S.H. plus L.H. is the most reliable ovulating mixture, it should be the preparation of choice for experimental ovulation studies. Ovulation was always followed by corpus luteum formation and pseudopregnancy. A further increase in the L.H. present in the intravenous mixture resulted in premature luteinization of smaller follicles with entrapped ova, and cystic degeneration of larger follicles. The number of ovulation points decreased progressively as the L.H. concentration was raised, ovulation rarely occurring when F.S.H. was combined with more than 200 mgm. eq. of L.H.

The intravenous injection of sheep L.H. after preliminary follicular development with F.S.H. was found by Casida (1) to be quite efficient

TABLE 3
Horse pituitary extracts

This group received a preliminary treatment of 2 r.u. of F.S.H. (60 mgm. eq.) daily for 5 days subcutaneously.

NUMBER OF ANIMALS	INTRAVENOUS INJECTION	NUMBER OVULATING	TOTAL NUMBER OF OVULATION POINTS
3	0.1 gm. (3 r.u.) F.S.H.	3	138
3	Same F.S.H. plus 10 mgm. eq. L.H. or 0.1 r.u. P.U.	3	414
2	Same F.S.H. plus 200 mgm. eq. L.H. or 0.5 r.u. P.U.	2	52
3	Same F.S.H. plus 1000 mgm. eq. L.H. or 2 r.u. P.U.	1	12

in eliciting ovulation of 8 to 40 points each in 10 out of 12 animals. We obtained similar results, but observed that administration of the L.H. intravenously at the same time the last subcutaneous injection of F.S.H. was made, resulted in augmentation and cystic degeneration with few if any ovulation points following such treatment.

Horse pituitary extracts produced results very similar to those obtained with sheep preparations. Again the purest F.S.H. employed, when injected intravenously following preliminary follicular development, resulted in superovulation. Addition of horse L.H. or pregnancy urine extracts to the intravenous injection of F.S.H. reduced this number gradually, but to completely prevent ovulation 1000 mg. eq. L.H. was required (see table 3).

Hog A.P. extracts were found to be unsatisfactory for experimental ovulation. Administration of F.S.H. (0.5 to 1 gm. eq., or 1-2 r.u.) daily for

5 days resulted in the development of pure follicles, but the ovaries were not large and the number of follicles stimulated was small. Intravenous injection of F.S.H. after preliminary follicular growth always failed to elicit ovulation. Addition of increasing amounts of L.H. did not give results similar to those obtained with sheep and horse preparations. Small amounts of L.H. plus F.S.H. intravenously gave an average of about 10 ovulation points. Increasing the L.H. concentration (table 4) did not alter this response until high dosages of L.H. were used, but as with sheep and horse extracts high dosages of L.H. completely inhibited ovulation.

By combining results obtained on all types of extracts it was found that intravenous injection of F.S.H. after preliminary follicular development did not always result in ovulation, that an intravenous injection of F.S.H. containing traces of L.H. gave superovulation with an average of 35 ovulation points, that with the addition of 10 to 20 mg. eq. of L.H.

TABLE 4

Hog pituitary extracts

This group received a preliminary treatment of 1-2 r.u. F.S.H. (0.5 to 1.0 gm. eq.) daily subcutaneously for 5 days.

NUMBER OF ANIMALS	INTRAVENOUS INJECTION	NUMBER OVULATING	TOTAL NUMBER OF OVULATION POINTS
4	0.5 gm. (0.5 to 1 r.u.) alone	None	
6	Same F.S.H. plus 10 to 20 mgm. eq. L.H., or 0.1 r.u. P.U.	6	42
4	Same F.S.H. plus 200 mgm. eq. L.H. or 1.0 r.u. P.U.	0	

to the intravenous injection of F.S.H. 18 to 21 ovulation points resulted. Addition of 100 mgm. L.H. or more to the F.S.H. gave large cystic follicles and corpora lutea but no ovulation points. Replacement of L.H. with pregnancy urine as an augmenting substance for the intravenous injection gave results very similar to those with F.S.H. and L.H.

In addition to the preceding experiments a group of 8 animals was treated with pregnant mare serum subcutaneously (2 to 20 r.u. daily) for 5 days, followed by an intravenous injection of the same extract. This resulted in an average of only two to three ovulation points each. The ovaries were not increased in size nor did the addition of small amounts of L.H. or P.U. to the intravenous mixture alter the above results.

Three animals were injected with P.U. subcutaneously but only a few follicles were stimulated. These became cystic after the intravenous injection. In none of these animals did ovulation occur.

DISCUSSION. The results of these experiments suggest that the development of the graafian follicle prior to the final stages of maturity is due to F.S.H. alone, for even small traces of L.H. added to F.S.H. and injected subcutaneously were sufficient to initiate preovulatory swelling and ovulation. The injection of F.S.H. which contained higher concentrations of L.H. resulted in injury to granulosa cells with cyst formation and premature luteinization. Experimental ovulation of follicles after such treatment invariably failed.

Experimental ovulation has been induced, after preliminary follicular development, by the intravenous injection of various preparations, but F.S.H. combined with traces of L.H. or P.U. gave the most consistent responses. This suggests that copulation in acyclic mammals accelerates the secretion of F.S.H. and at the same time releases small amounts of L.H. which acts synergistically with F.S.H. resulting in luteinization and ovulation.

The results obtained with F.S.H. extracts intravenously, although suggestive, are not final. After many tests on immature rats and adult oestrous rabbits, and careful study of ovarian histology as modified by these extracts, it was found that the majority of preparations contained minute traces of L.H. Recent unpublished observations have demonstrated that the purest F.S.H. available is capable of inducing ovulation in both juvenile and adult rabbits, but the dosage is many times that of F.S.H. plus L.H., and the number of ovulation points is very small.

The comparative effectiveness of L.H. from different sources in overriding the ovulation response when the concentration was raised in the intravenous mixture varied greatly on the basis of dry pituitary powder weight, but on the basis of actual ovulating capacity as demonstrated in the adult oestrous female (unpublished observations) the amount was practically the same. The mechanism of this inhibition by large amounts of L.H. was primarily one of overstimulation which resulted in atresia and premature luteinization.

SUMMARY

1. A study of artificial follicular development in the juvenile rabbit by subcutaneous injection of follicle-stimulating pituitary extracts (F.S.H.) alone and combined with increasing amounts of luteinizing pituitary extracts (L.H.), revealed that F.S.H. was the only preparation capable of inducing pure follicular development. Such follicles were normal as shown by the fact that intravenous injection of an ovulating mixture resulted in ovulation, corpus luteum formation and pseudopregnancy.

2. The majority of animals treated with F.S.H. plus traces of L.H. subcutaneously ovulated spontaneously. Intravenous injection was ineffective in increasing the number of ovulation points.

3. Treatment with F.S.H. and higher concentrations of L.H. resulted in atresia, cystic degeneration and luteinization. Experimental ovulation under these conditions always failed.

4. Following adequate follicular development by F.S.H., the intravenous injection of F.S.H. alone induced ovulation in many instances, but was not uniformly reliable. However, F.S.H. plus traces of L.H. always resulted in ovulation. The number of ruptured follicles varied with the preparation employed. Using sheep preparations the average number of ovulation points was 22, while it averaged 38 for the horse and only 7 for hog preparations. By gradually increasing the L.H. in the intravenous mixture (F.S.H. dosage constant) the number of ovulation points steadily decreased until ovulation failed as higher concentrations of L.H. were reached.

5. Extracts of pregnant mare's serum injected subcutaneously were found to stimulate only a small number of follicles in the immature rabbit. The intravenous injection resulted in ovulation of only two to four follicles without increasing ovarian size.

6. Pregnancy urine preparations (P.U.) were unsatisfactory for follicular development, but could be satisfactorily substituted for L.H. in the intravenous mixture.

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THE DIFFERENTIAL ACTION OF PITUITARY GONADOTROPIC HORMONES UPON THE SECRETORY CAPACITY OF THE GRAAFIAN FOLLICLE AND CORPUS LUTEUM

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Although histological changes in the genital tract resulting from administration of the pituitary sex hormones are well known, few studies have been made in which spontaneous motility and pituitrin reactivity of the uterus have been utilized as a means of estimating the secretory activity of the corpus luteum or the graafian follicle under hormonal stimulation. The present investigation is a study of the differential action of the follicle stimulating (F.S.H.) and luteinizing (L.H.) pituitary hormones upon isolated and pseudopregnant rabbits in which uterine motility and histology have been used as a measure of oestrin and corpus luteum secretion.

METHOD. Based on the assumption that spontaneous motility with maximal reactivity to pituitrin indicates oestrin secretion, and that inhibition of spontaneous motility with pituitrin refractivity results from corpus luteum secretion, motility reactions of uteri have been studied following subcutaneous administration of (1) F.S.H. extracts, (2) L.H. extracts, (3) F.S.H. mixed with various amounts of L.H., and (4) F.S.H. followed by L.H. The uteri of immature rabbits have also been studied after experimental ovulation to determine the duration of the resulting pseudopregnancy in 1, untreated animals; 2, animals treated with F.S.H., and 3, those treated with L.H.

The bath fluid which was prepared according to the method of Van Dyke and Hastings (11), consisted of a carbonate-phosphate buffered saline solution containing the inorganic constituents of blood serum and at the same pH. The apparatus was suspended in a constant temperature oven at 37.5°C. with the pH and CO₂ tension maintained by bubbling the oxygen supply through sodium bicarbonate solutions. The bath fluid was freshly made from stock solutions for each test. The motility chamber contained 50 to 70 cc. of fluid.

Juvenile rabbits 12 to 14 weeks of age weighing 1200 to 1400 grams were used. Extracts of F.S.H. and L.H. were prepared from dried sheep

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pituitary powder by the methods used in this laboratory (3). Uterine biopsies were removed at intervals of three to five days for motility tests as well as histological sections. The dosages employed in these experiments together with motility and pituitrin reactions are presented in table 1.

Action of pituitary gonadotropic hormones on graafian follicle and corpus luteum. The first group of 18 animals treated with F.S.H. exhibited maximal spontaneous motility and pituitrin sensitivity. The single exception was one animal which had not reached the stage of maturity compatible with maximal responsiveness to F.S.H. The histological

TABLE 1
Spontaneous uterine motility and pituitrin sensitivity after subcutaneous injection of various pituitary preparations

GROUP	EXTRACT AND TOTAL DOSAGE INJECTED OVER A PERIOD OF FIVE DAYS	NUMBER OF ANIMALS	SPONTANEOUS MOTILITY PRESENT	PITUITRIN RESPONSE POSITIVE*
1	2 to 4 gm. F.S.H. (8 to 18 r.u.)	18	17	18
2	2 gm. L.H.	10	1	0
3	2 to 4 gm. F.S.H. plus small amounts of L.H.	9	9	5
4	2 gm. crude A.P. (10 r.u.)	22	12	7
5	2 gm. F.S.H. subcut. 5 da. followed by 2 gm. L.H. over a period of 5 da.	23	10	11
6	Artificial pseudopregnancy resulting from experimental ovulation	10	2	0
7	Pseudopregnant animals treated with 2 to 5 gm. F.S.H. over 5 days	7	6	7
8	Pseudopregnant animals treated with 2 to 5 gm. L.H. over a period of 5 days	9	2	3

Each figure represents number of animals showing a given reaction.

* Dosage of pituitrin 0.01 to 0.03 cc. U.S.P. in 50 cc. bath. All uteri were tested with 0.1 cc. 1:1000 solution of epinephrine in 50 cc. bath to determine maximal motility responses.

appearance and motility of these uteri were identical with those of animals receiving 50 to 200 r.u. of oestrin over a five day period.

In striking contrast to the above results the administration of L.H. subcutaneously resulted in luteinization and maximal progestational changes with absent spontaneous motility and loss of pituitrin responsiveness.

Treatment with F.S.H. containing small amounts of L.H. resulted in corpus luteum formation, and ovulation in some animals. The uteri were pseudopregnant with plus one to plus four reactions, yet spontaneous motility was present and pituitrin inhibition occurred in only four animals.

Further increase in the concentration of L.H. as in crude A.P. extracts resulted in loss of spontaneous motility in 10 out of 22 animals, while 15 failed to respond to pituitrin. All animals with solidly luteinized ovaries were found to have uteri which did not respond to pituitrin or exhibit spontaneous motility. Uteri from those in which luteinization was irregular, with ovaries containing cystic and hemorrhagic follicles as well as corpora lutea, responded to pituitrin and usually the progestational reaction was less marked than that which accompanied solidly luteinized ovaries.

A group of 23 animals receiving F.S.H. three to five days followed by L.H. over a similar period responded by luteinization, cyst formation, and ovulation in 9 animals. The uteri showed progestational changes, and over half of the group had no spontaneous motility while a similar number failed to respond to pituitrin.

The uteri of animals experimentally ovulated by methods previously described (4) had no spontaneous motility (2 exceptions) and failed to respond to pituitrin. When F.S.H. was administered, spontaneous motility and pituitrin sensitivity returned, although the endometrial changes did not regress so rapidly. Pseudopregnant animals treated with L.H. showed a tendency toward loss of pituitrin inhibition, but this did not usually manifest itself in five days.

The effect of the pituitary sex hormones on the life of the corpus luteum. In order to use the immature rabbit for experimental study of the functional life of the corpus luteum, it was first essential to determine the normal limits of experimental pseudopregnancy. A group of eight animals was used for this study. Experimental ovulation was induced (10 to 50 ovulation points) by methods previously described (4), and uterine biopsies were removed at intervals of three to five days. These were studied histologically and by motility reactions. The pseudopregnant period lasted from 12 to 15 days, with an average of about 13.8 days, which is shorter than that reported for the adult (9). The usual signs of corpus luteum regression were a return of spontaneous motility and pituitrin sensitivity. These appeared on about the 10th day in most instances.

Seven immature rabbits were ovulated experimentally and then treated with F.S.H. (1 to 1½ gm. daily, 3 r.u.). Biopsies were removed at intervals of two to three days. The length of corpus luteum activity in this group was found to vary from two to seven days. Motility reactions did not show the usual absence of spontaneous motility and pituitrin inhibition. One rabbit which had 60 ovulation points showed marked cystic hyperplasia of the endometrium in seven days. Two animals failed to show morphological evidence of pseudopregnancy, while the others demonstrated maximal pseudopregnancy changes with marked sensitivity to pituitrin and considerable spontaneous motility at five days after ovulation. Further treatment resulted in complete regression to an oestrous

endometrium. Ovarian histology showed atypical luteinization and cystic follicles.

Eight pseudopregnant immature rabbits were treated with 0.5 to 1.0 gram of L.H. subcutaneously daily. The duration of corpus luteum activity was 7 to 10 days (average 9 days). Spontaneous motility varied with the dosage, being absent with high dosages and present with lower dosages. In spite of the absence of spontaneous motility, two of the uteri showed maximal pituitrin reactivity in the presence of plus four endometrial proliferation. In most instances morphological regression preceded loss of pituitrin inhibition. The ovaries in these animals showed a gross reduction in size with blanching of the corpora lutea. Microscopic

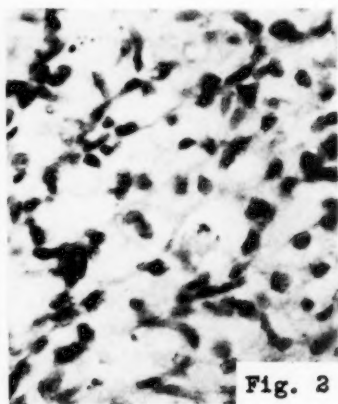
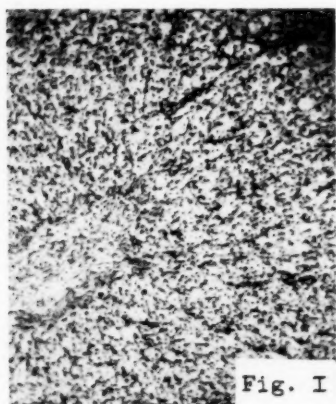


FIG. 1. A low power photomicrograph of a corpus luteum after administration of L.H. showing fibrosis.

FIG. 2. A high power magnification of the same demonstrating the nuclear achromatolysis and vacuolar degeneration resulting from L.H. treatment.

examination revealed vacuolation and nuclear achromatolysis of the lutein cells with some fibrosis (fig. 1). Accompanying these changes marked follicular atresia was noted.

These observations on immature rabbits during experimental pseudopregnancy were also extended to adult mated does. One group of four animals was injected with F.S.H. and another with L.H. in the same dosages as employed in the immatures. The group receiving F.S.H. showed evidences of corpus luteum activity for 2 to 13 days with an average of 7, but failed in all instances to develop implantation sites. One of the group did not develop progestational changes in the endometrium, and luteinization was apparently interrupted. Another animal had the follicles cauterized 6 days after mating to determine whether overstimulation of the follicles by F.S.H. was a factor in overthrowing

hormone balances essential for morphological changes characteristic of pregnancy. This animal began implantation, but resorbed almost immediately. Pregnancy changes were present for 13 days (7 days after treatment was begun) which suggested that the follicles were not essential for this termination of lutein activity. Active spontaneous motility and marked pituitrin sensitivity were present as observed in the immatures. The L.H. treated animals maintained endometrial changes of pregnancy for about 7 days. Pituitrin inhibition faded with the regression of the endometrium. The ovaries showed fibrosis and degeneration of the lutein cells with the uniform development of large cysts. Only one animal implanted, but resorption followed promptly. It would seem that the conditions obtaining as a result of F.S.H. or L.H. administration are incompatible with normal implantation.

DISCUSSION. The administration of oestrin results in uterine enlargement with spontaneous motility similar to that observed in oestrous adults, and as in the latter, marked sensitivity to pituitrin obtains. This identical situation is observed after administration of F.S.H. which makes it apparent that F.S.H. acts by stimulating graafian follicles to secrete oestrin thus transforming a non-motile threadlike uterus into the large vascular structure of full oestrus.

Earlier studies (10) indicated that a preliminary oestrous condition was essential for endometrial modification by the corpus luteum hormone. However, more recent observations prove that high dosages of progesterone will produce typical progestational changes in adult female rabbits castrated for months (8). These observations may explain why L.H. administered to untreated immature rabbits with small atrophic uteri is capable of inducing changes characteristic of high doses of progesterone with loss of spontaneous motility and marked pituitrin inhibition.

In general, combinations of F.S.H. and L.H. induced reactions falling between the extremes of either fraction alone (F.S.H. resulting in increased spontaneous motility and pituitrin sensitivity, and L.H. resulting in a loss of spontaneous motility and pituitrin sensitivity). F.S.H. plus small traces of L.H. induced spontaneous ovulation, but no inhibition to pituitrin. F.S.H. plus larger traces of L.H. entrapped ova by rapid luteinization which prevented ovulation. The uteri did not respond to pituitrin. When still larger amounts of L.H. were injected, spontaneous motility faded as did the sensitivity to pituitrin.

In the animals treated with L.H. (injected subcutaneously as a neutral powdered emulsion) following a previous treatment with F.S.H. absorption was retarded and frequently the ovaries did not luteinize uniformly; but as in the injection of crude A.P., when luteinization was uniform, pituitrin inhibition and loss of spontaneous motility always occurred.

The action of F.S.H. in reducing experimental pseudopregnancy was

probably due to oestrin secretion, for it has been demonstrated (1), (2) that oestrin treatment will override the action of normal corpora lutea without actual injury to lutein tissue. Zondek (12) has recently reported degenerative changes in the corpora lutea of normal women injected with 50,000 m.u. of oestrin daily, but this may have been due to the release of L.H. The main source of oestrin under F.S.H. stimulation is probably the graafian follicle, but the evidence of oestrin secretion in solidly luteinized ovaries or those with cauterized follicles suggests that the corpora lutea may secrete oestrin also. The fact that oestrin is present in normal lutein tissue supports this hypothesis.

L.H. evidently shortens the duration of experimental pseudopregnancy and the life of the corpus luteum by overstimulation which eventually results in injury, as is suggested by the vacuolation and fibrosis of lutein cells accompanied by the regression of progestational changes, loss of pituitrin inhibition and return of spontaneous motility. Others have previously demonstrated (7) the deleterious effects of L.H. on corpora lutea of hypophysectomized rats. These authors showed that L.H. treatment resulted in almost complete involution of corpora lutea which in the controls remained unchanged for weeks. Thus, there are probably two ways of overthrowing the hormonal balance necessary for the pseudopregnancy: 1, by oversecretion of oestrin through F.S.H. stimulation, and probably inhibition of luteinization (5) when F.S.H. is present in large quantities at the time of ovulation; 2, by direct injury to lutein cells as a result of overstimulation with large doses of L.H.

The explanation of the loss of pituitrin inhibition with maximal pseudopregnancy changes present, as resulted from treatment with certain combinations of F.S.H. and L.H., would seem to be due to the oestrin-corpus luteum balance. We have recently (6) duplicated this picture with a combination of 4 Rb. U. progesterone plus 150 r.u. of oestrin injected over a 5 day period.

SUMMARY

1. Administration of F.S.H. to juvenile rabbits apparently stimulated the graafian follicles to secrete oestrin which resulted in spontaneous motility and marked pituitrin sensitivity of uteri normally non-motile.

2. L.H. treatment resulted in luteinization and corpus luteum hormone secretion with the uterus developing a motile state characteristic of pseudopregnancy (progestational endometrial reaction, absent spontaneous motility, insensitivity to pituitrin and maximal response to adrenalin).

3. F.S.H. plus a trace of L.H. resulted in spontaneous ovulation, but larger doses of L.H. were necessary before inhibition to pituitrin developed. Further increase in L.H., when luteinization was uniform, always

resulted in pituitrin inhibition and in most instances loss of spontaneous motility.

4. Artificial pseudopregnancy following experimental ovulation lasted 13 to 14 days in juvenile rabbits. Injection of F.S.H. shortened this period to six days or less, and L.H. injections limited it to nine days. F.S.H. apparently acted through oestrin secretion and inhibition of luteinization, while L.H. acted through overstimulation and injury to lutein tissue.

5. Mated adult females injected with F.S.H. or L.H. in the same dosages failed to implant. Progestational changes lasted 2 to 13 days and motility reactions were similar to those described in the immature animals.

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THE RELATIONSHIP BETWEEN ABDOMINAL, UTERINE AND ARTERIAL PRESSURES DURING LABOR

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The earlier literature on the measurement of intra-uterine pressure has been reviewed fully elsewhere (1, 2, 3). Some investigators have deduced the pressures developed during labor from the force necessary to rupture the membranes (4), others from the force apparently exerted upon the baby (5). The classical attempt at direct measurement is that of Schatz (1) who, in 1872, registered the pressure exerted by the abdominal and uterine walls upon a bag placed in the uterine cavity. Pressure was transmitted through a column of water to a mercury manometer, and records were presented of the labor pains of many women in normal labor (without anesthetic), and in one case under chloroform. Using the same apparatus the effect of other drugs was investigated by Hensen (3).

The records which we present parallel closely those of Schatz and Hensen as to intra-uterine pressure, but by the use of a differential manometer we are able to present also the contribution separately of the uterine and abdominal walls to the intra-uterine pressure, the changes in the maternal systemic blood pressure during labor, and the result of uterine contractions upon the effective head of maternal arterial pressure to the placenta.

METHOD. The manometer is a development of the hypodermic manometer previously described (6) so modified as to record differences in pressures rather than simple pressures (7). The pressure is optically recorded in an electrocardiographic camera by the tipping of the mirror, *M* (fig. 1), which is eccentrically supported upon a thin silver diaphragm. This diaphragm bulges one way or the other, depending upon whether the pressure is greater in the chamber in front of the diaphragm or in the tube behind the diaphragm. The deflection of the mirror is directly proportional to the algebraic sum of the pressure in the tube (+) and the pressure in the chamber (-).

At the end of every experiment the manometers were calibrated by connecting them to known pressures measured with a mercury manometer. The deflections were photographed and a calibration curve constructed from which the experimental records could be measured to the nearest

mm. Hg. The patient was usually one whose pregnancy had best be terminated. It is the custom in this hospital to induce labor by introducing a catheter into the uterus outside the chorion on the side opposite the placenta. In our cases a soft rubber balloon (condom) was tied over the end of the catheter and a lead bougie inserted temporarily into its lumen. With the catheter thus stiffened it was easily introduced into the fundus of the uterus and its final position checked by x-ray.

A similar balloon was tied over the end of a duodenal tube and introduced into the stomach by way of the nose. As soon as labor began the patient was sent to the laboratory and the pressure records taken as described below.

Physiological pressure relationships. Pressure changes in a labor pain occur as shown in figures 2 and 3 (values given in table 1). The upper record is taken with a simple manometer, connected with a needle inserted into the brachial artery. The peak of each individual pulse beat determines the systolic, the trough, the diastolic pressure and each can be measured accurately with the calibration curve.

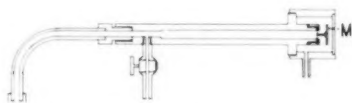


Fig. 1. An optical differential manometer. *M* is a mirror supported by a thin silver diaphragm which bulges one way or the other, depending upon whether the pressure is greater in the chamber in front of the diaphragm or in the tube behind the diaphragm.

During the pain the arterial pressure rises. In many pains the pulse pressure widens as well (see figs. 8 and 9). These changes are probably due to the fact that placental blood is forced out into the systemic circulation. In addition to this steady rise (fig. 2) there are waves in the arterial pressure, due to changes in intrathoracic pressure, which are propagated out the arterial tree. These appear as small respiratory waves early in the pain when the patient was groaning restlessly and as a large sustained rise in the middle of the pain when the patient was told to "bear down."

The third curve from the top was also recorded by a simple manometer. It records the total intra-uterine pressure from a balloon in the fundus uteri. The pressure waves seen in the arterial pressure record are also seen in the record of the intra-uterine pressure. They are the effects of contracting the skeletal muscles of the abdominal and thoracic walls which add to the pressure within the uterus. The strong bearing down effort at the middle of the pain nearly doubles the intra-uterine pressure.

The lowest curve was taken with a differential manometer. Pressure

from the gastric balloon was led to the front chamber and from the uterine balloon was led to the tube behind the silver membrane supporting the mirror. The mirror records then the difference between the intra-gastric and intra-uterine pressure. We interpret this physiologically as the contribution of the uterine walls to the intra-uterine pressure, and shall refer to it as the "uterine component." The pressure changes produced

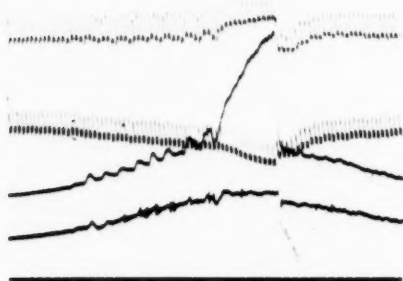


Fig. 2

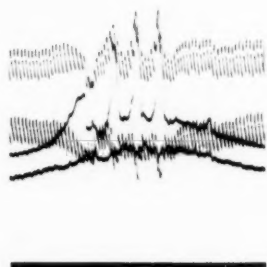


Fig. 3

Figs. 2, 3. Pressure curves during labor (fig. 2, early, fig. 3, late in labor). Pressure curves are defined from above downward—the brachial blood pressure (*B. B. P.*); the maternal placental blood pressure (*P. B. P.*) (*B. B. P.* minus the intra-uterine pressure); the intra-uterine pressure (*I. P.*); and the uterine component of the intra-uterine pressure (*U. C.*) (*I. P.* minus the gastric pressure).

TABLE I

The effect of labor pains on the intra-uterine pressure, the uterine component, the brachial blood pressure, and the effective placental blood pressure (mm. Hg)

	FIG. 2				FIG. 3			
	I. P.	U. C.	B. B. P.	P. B. P.	I. P.	U. C.	B. B. P.	P. B. P.
Before pains	20	10	90/50	70/30	25	20	150/100	125/75
Height of pain and before "bearing down"	65	45	95/60	30/-5	55	50	150/100	95/45
While "bearing down"	120	50	120/83	0/-37	160	50	205/165	45/5
Immediately after ceasing to "bear down"	65	45	65/43	0/-23	55	50	140/100	85/45
Three seconds later	50	40	95/65	45/15	55	50	150/110	95/55

by the abdominal muscles are eliminated because they make pressures which are led to both sides of the sensitive membrane. This leaves the uterine component, a symmetrical smooth-muscle contraction curve, reaching heights in various experiments of from 25 to 95 mm. Hg.

The second curve from the top is also traced by a differential manometer. The tube of the manometer is connected by lead tubing to the same needle as was used in taking the upper curve. The balloon in the

uterus was connected to the chamber in front of the sensitive membrane. This curve therefore records the difference between the blood pressure and the intra-uterine pressure. We interpret it physiologically as a record of the effective head of maternal arterial pressure to the placenta. This pressure is somewhat lower between pains than is the systemic blood pressure because of the tonic activity and elastic recoil of the uterine and abdominal muscles. As the uterus contracts there is a steady lowering of the placental head in spite of the fact that increased venous return from the squeezed placenta, among other factors, is increasing the general level of the systemic arterial pressure.

It will be noticed that the minor waves of arterial pressure, which are produced by skeletal contractions of the abdominal or thoracic muscles, are eliminated from this record of "effective placental blood pressure."

It has been shown elsewhere (8) that efforts of short duration, such as coughing, which raise the intrathoracic pressure, raise also in identical fashion the intraspinal, abdominal and arterial pressures. The net blood pressure to these organs remains constant. This insures continued irrigation of the vital tissues and at the same time guards against strain upon the arteries supplying the vital organs within these cavities. The peripheral arteries of the systemic system are not protected by external pressure against these strains. Small conjunctival and subcutaneous hemorrhages are common in childbirth. Similar effusions in the organs of the abdomen, thorax and cerebro-spinal canal, are correspondingly rare.

An increase of longer duration, such as is shown in the bearing down effort in figure 2, results in a parallel increase in the thoracic abdominal and spinal pressures. The arterial pressure starts up in a similar course but is interrupted by the fact that the high intrathoracic pressure hinders venous return to the heart, decreases the pulse pressure and limits the rise of mean arterial pressure (8). At the end of the effort the blood pressure is no longer supported by the intrathoracic pressure, and suddenly drops off to an abnormally low level. Increase to normal (or above) in the venous return brings back to normal (or above) the systemic arterial pressure.

In many cases the intra-uterine pressure rises up to or even above the systolic pressure. If a "bearing down" effort is long continued or very strenuous this is particularly apt to occur. When it does occur there is no effective arterial pressure to the placenta. Blood is even being forced back through the uterine arteries to the aorta (see table 1). This is guarded against by nature in three different ways: 1, by a moderate normal increase in arterial tension in women before childbirth; 2, by an increase in arterial tension during each pain and during each effort at bearing down, if these are not prolonged; 3, by the short duration of the natural reflex efforts at bearing down. They are usually so short that they do not materially hinder venous return to the heart.

In figure 3 the pain illustrated was late in labor. The bearing down

efforts were reflex and of short duration. The blood pressure was somewhat elevated and the placental pressure head was at all times sufficient to insure adequate irrigation of the placenta in spite of the fact that the intra-uterine pressure went up to the figure of 160 mm. Hg.

The above method of recording was used in a majority of the observations—figures 2, 3, 7, 8, 9, 10, 11, 12 and 13, in figure 6 the blood pressure tracings were omitted; and in figures 4 and 5, the balloons were so arranged as to determine the difference between pressures in the cervix below the head and in the fundus above the baby.

In figure 4 (a record from the first stage of labor), the upper tracing is the pressure developed in the uterus above the fetus. It is a simple uterine contraction with a bearing down effort superimposed. The lower

Fig. 4. Record taken early in the first stage of labor. The upper tracing of the intra-uterine pressure, *I. P.*, recorded a tone of 20 mm. Hg between pains. This increased to 80 mm. during the pain. "Bearing down" caused a further increase to 120 mm. The middle tracing recorded the expulsive pressure, *E. P.* (fundic pressure minus cervical pressure) which remained near 0. Bottom tracing is the base line.

Fig. 5. Record similar to figure 4, which was taken late in the second stage of labor. Between pains the *I. P.* was 18 mm. Hg, the *E. P.* was 15 mm. At the height of the pain the *I. P.* was 60 mm., the *E. P.* was 50 mm. "Bearing down" increased the *I. P.* to 150 mm. and *E. P.* to 140 mm.

Fig. 6. Two records taken during the delivery of the head. In each record the upper curve recorded the intra-uterine pressure, the middle curve the uterine component, *U. C.*, and the lower line is the base line. Upper record—from a patient with strong pains; at the height of the pains the *I. P.* reached 215 mm. Hg and the *U. C.* was 90 mm. Lower record—from a patient with weak pains; the *I. P.* reached 130 mm. and the *U. C.* was 40 mm.

Fig. 7. Effect of a barbiturate and hyoscine during the first stage of labor. Upper record—before administering, lower record—twenty minutes after administering 0.4 gram of pentobarbital-sodium and 0.4 mgm. of hyoscine hydrobromide. The curves are the same as in figure 2. Average values for these records are given in table 2.

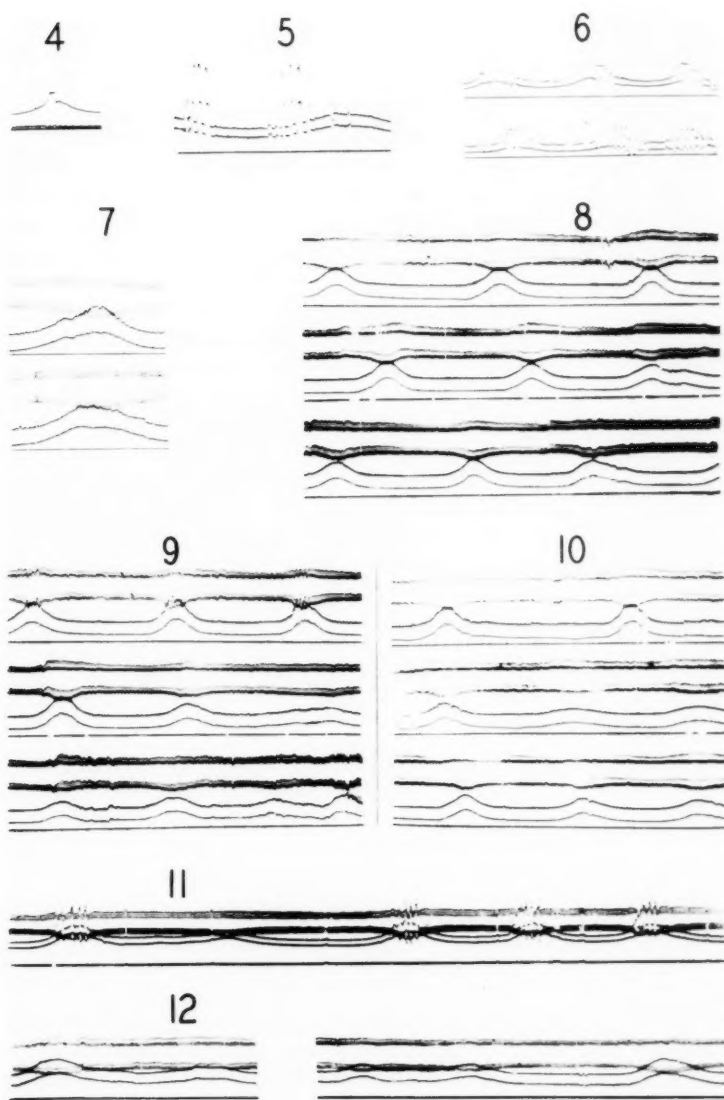
Fig. 8. Effect of nitrous oxide-oxygen anesthesia during the first stage of labor. Upper record—before, middle record—during, and lower record—immediately after nitrous-oxide-oxygen anesthesia. The curves are the same as in figure 2. Average pressure values are given for these curves in table 2.

Fig. 9. Effects of ether anesthesia during the second stage of labor. Records are similar to figure 8 except that ether was administered during the pains in place of nitrous oxide-oxygen. Average pressure values are given in table 2.

Fig. 10. Effects of chloroform anesthesia during the first stage of labor. Records are similar to figure 8. Average pressure values are given in table 2.

Fig. 11. Effects of cyclopropane anesthesia during the second stage of labor. The record shows pressure curves of one normal pain, of one pain while the patient was deeply anesthetized with cyclopropane, and of three pains following the withdrawal of the anesthesia. Average pressure values are given in table 2.

Fig. 12. Effects of morphine sulfate during the first stage of labor. These records were taken just before and twenty minutes after administering 15 mgm. of morphine sulfate. Average pressure values are given in table 2.



Figs. 4-12

record, just above the base line, is the difference between the fundic and cervical pressures, i.e., the expulsive pressure. This record, which was taken before any cervical dilatation had occurred, indicates that in the earlier stage pressure is distributed throughout the uterus. The fact that the pressure is as high in front of the baby as behind does not indicate that the cervix was not slowly dilating, because it was. No doubt it was slowly giving in to the pressure from behind. Nor does the distribution of pressure shown in the record invalidate the observations of Ivy et al. (9) of the peristaltic action of the uterus. The earlier fundic contractions increase pressure instantaneously over the whole of the contents of the distended uterus.

The fact that abdominal effort produced no expulsive force shows that the pressure thus produced was communicated to the structures in front of the baby to the identical degree as to the structures behind. Such a force, acting from outside the uterus can, of course, produce no dilatation of the cervix and cannot further the course of labor. It is interesting to recall that there is no reflex or natural "bearing down" in this stage.

Figure 5 shows the same sort of record taken after cervical dilatation. The bearing down efforts are naturally evoked several times during each labor pain. They are effective in contributing to expulsion because the pressure below the baby does not rise and the record measuring the "expulsion pressure" goes up not only with uterine contraction but also with the bearing down efforts.

Multiplying the area of the cross section of the child's head by the pressures acting upon it, we have calculated, in seven cases, the force in kilograms which the uterus and abdominal muscles each exert in pushing the baby out. During the time of the delivery of the head the uteri contributed respectively 4, 7, 7, 7, 7, 8 and 8 kgm. upon the heads, while the abdominal muscles increased these expulsive forces to 12, 11, 11, 19, 19, 10 and 21 kgm., respectively.

In figure 6 are records from the first (lower record) and last (upper record) cases which represent the extremes of the uterine contributions. In the lower record, where the uterine pains were weak, delivery was accomplished through greater aid from the abdominal muscles.

During the delivery of the head the contracting uterus subjected itself to a maximal pressure (average of 6 cases) of 100 grams per square centimeter. By a method to be described elsewhere the volume of the uterus was measured. With these figures, assuming that the contracting uterus was spherical, the force tending to rupture the uterus was calculated.

This force gradually increased as labor progressed and during the second stage reached an average of 250 grams and a maximum of 300 grams per centimeter on the uterine circumference. Since the average thickness of the wall of a full term uterus measured 5 mm., the maximal tension

was approximately 600 grams per square centimeter of cross sectional area of uterine wall.

The effects of certain anesthetics and of epinephrine. Whereas none of the anesthetics changed the frequency or duration of the uterine contractions, they had the varying effects detailed below upon the strength of the contractions and upon the contraction of the auxiliary abdominal muscles.

In figure 7 records taken before and twenty minutes after the administration of pentobarbital-sodium and hyoscine show that these drugs inhibited the natural bearing down efforts and that the uterine contractions remained normal in frequency, duration and strength. The inhibition of the bearing down efforts was perhaps merely a delay because these came on under the same drug later in labor (see fig. 6). The blood pressure values remained within normal limits.

In figure 8 the top row of tracings was taken before any drug was given, the middle row was taken with the patient under full nitrous oxide-oxygen anesthesia, and the lower row during recovery. The blood pressure, the strength, duration and frequency of the uterine contractions remained within normal limits.

The same experiment was repeated with ether, see figure 9. Strong reflex bearing down efforts that affected the pressures in the upper row of tracings were stopped by ether, as shown by the smooth intra-uterine pressure curves in the middle row. These same curves show a marked falling off of the uterine response. The return to normal, see lower row, is delayed. When ether was first administered the blood pressure rose temporarily 5 mm. Hg.

Similar effects are seen with chloroform administered earlier in labor (see fig. 10) except that the blood pressure values decreased 5 mm. Hg.

Cyclopropane administered during the second pain (in fig. 11) caused a more complete elimination of skeletal muscular movements, but only a small interference with the uterine contraction and no change in the blood pressure. Recovery is seen to be very rapid.

Records in figure 12 taken before and twenty minutes after administering 15 mgm. of morphine sulfate early in labor show that this drug caused very little effect upon either the blood pressure or the uterine contractions. Strong "bearing down" efforts were not yet present in this patient.

It is of practical importance to note that though the anesthetics prolong labor, reducing the intra-uterine pressure either by acting on the uterus or on the abdominal wall, in the same measure they increase the effective placental blood pressure and avert the danger of fetal asphyxia (see table 2).

The effect of intravenous administration of 0.1 mgm. epinephrine hydrochloride (see fig. 13) is to produce a marked rise in blood pressure and intra-uterine pressure. The uterine contraction is stronger and much

more abrupt in onset than the normal contractions. The rise in blood pressure, however, is greater than the increase in intra-uterine pressure so that ample blood pressure is supplied to the placenta during the response. The relaxation is slower if anything than normal and followed by two quickly succeeding smaller contractions. After this response uterine excitability seems somewhat lessened to return soon to normal.

TABLE 2

Effects of anesthesia upon the intra-uterine pressure, the uterine component of the intra-uterine pressure, the brachial blood pressure, and the effective placental blood pressure during pains (mm. Hg)

	MAXIMAL INCREASE IN PRESSURE DURING PAINS						B. B. P. BEFORE PAINS		MINIMAL P. B. P. DURING PAINS	
	Normal		Anes- thetized		Post anes- thetic		Normal	Anes- thetized	Normal	Anes- thetized
	I. P.	U. C.	I. P.	U. C.	I. P.	U. C.				
Fig. 7. Pentobarbital and hyoscine	70	48	46	46			150	85	145	90
Fig. 8. Nitrous oxide- oxygen	60	57	50	50	47	45	152	100	152	96
Fig. 9. Ether	110	59	35	35	31	25	145	95	151	98
Fig. 10. Chloroform	65	51	31	28	25	22	150	95	145	95
Fig. 11. Cyclopropane	155	40	39	36	150	38	135	90	135	90
Fig. 12. Morphine	60	39	50	41			138	80	140	80



Fig. 13. Effect of epinephrine. The signal on the left indicates the intravenous injection of 0.1 mgm. epinephrine HCl. The B. B. P. increased from 175-190 to 300-200 mm. Hg. The P. B. P. increased from 130-70 to 225-125 mm. The I. P. reached a maximal height of 180 mm. and the U. C. reached a maximal height of 80 mm. Note the cardiac irregularities following the injection.

The following have aided in the above investigation: Drs. R. B. Crichton, A. K. Temples, H. D. Frech, Jr., J. D. Gray, and M. B. Hatcher. It is a pleasure to record our thanks for their coöperation.

SUMMARY

1. By means of a set of differential manometers simultaneous records were taken during normal human labor pains of 1, the systemic arterial

pressure; 2, the effective head of maternal blood pressure to the placenta; 3, the total intra-uterine pressure; 4, the contribution of the uterine wall to the intra-uterine pressure.

2. Human uterine contractions result in smooth symmetrical pressure rises which vary in height from 25 to 95 mm. Hg and subject the uterine wall to an average maximal tension of 500 grams per square centimeter of uterine wall cross area. During delivery of the head, the average maximal expulsive force was 15 kgm. The frequency and duration of uterine contractions were unaffected by morphine, nitrous oxide-oxygen, the barbiturates, cyclopropane, ether and chloroform. The strength of the uterine contractions, markedly reduced by ether and chloroform, were unaffected by the other anesthetics. All of the above drugs reduced the contribution of the abdominal muscles to the intra-uterine pressure.

3. During labor pains the systemic blood pressure increases and the pulse pressure widens. The effective maternal arterial pressure to the placenta diminishes—sometimes to zero. The pressure which irrigates the placenta is increased by obstetrical anesthetics and by epinephrine.

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HOMEOPLASTIC TRANSPLANTATION OF ADRENAL GLANDS IN RATS OF INBRED STRAINS

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The adrenal glands of adult rats have been grafted autogenously with relatively consistent success but it is rare to obtain viable grafts of adrenal glands transplanted to unrelated animals. Jaffe reported successful homeografts in four animals out of fifteen. Wyman and Tum Suden (14) obtained viable homeografts when glands were exchanged between litter mates. More recently Wyman and Tum Suden (15) secured successful homeografts of adrenal glands exchanged between animals of their strain. Higher percentages were obtained with female than with male rats. Martin demonstrated successful intra-ovarian homeografts of the adrenal glands in three rats. Adrenal glands of new born rats, however, may be grafted to unrelated, adrenalectomized adult rats and a high percentage of them will regenerate and function (2, 3, 8).

The studies of Loeb (5, 6), over a number of years, on the individuality of tissues give strong support to the opinion that the genetic relationship between donor and host is the important factor in the survival and function of a graft. Loeb (6) has shown that the lymphocyte detects fine degrees of dissimilarity between donor and graft and that its presence in a graft indicates an antagonistic relationship which may ultimately destroy the graft. :

In this laboratory we have made homeografts of the adrenal gland in a large number of rats of our strain, not closely inbred, but have secured only a few functional grafts. Thus it has seemed of interest to make homeografts of the gland within strains of rats which we knew had been closely inbred for many generations, and thus were as nearly homozygous as possible. A report of a preliminary study was made by Nilson and Ingle.

METHODS. The animals used in these experiments were of two strains of piebald rats which had been developed from a common stock by selective inbreeding. The basis of selection was the relative efficiency of the rats in the utilization of their food. The reader is referred to the papers by Palmer and Kennedy and by Morris, Palmer and Kennedy for dis-

cussions of efficiency in utilization of food. The high efficiency strain had been inbred for twenty generations and the low efficiency strain for eighteen generations when our study was undertaken. Burhoe (1) recently has found that rats can be classified in four blood groups. He kindly tested these strains and found that rats of the high efficiency strain belonged to his AB group, while those of the low efficiency strain belonged to the O group.

Four series of experiments were performed. In the first series both adrenal glands were exchanged between twelve pairs of adult sisters within the high efficiency strain. In the second series, the adrenal glands were exchanged between nine pairs of adult distant cousins, also within the high efficiency strain. In the third series the adrenal glands were exchanged between seven pairs of adult sisters within the low efficiency strain. And in the fourth series the adrenal glands were exchanged between thirteen pairs of animals; one of each pair was of the high efficiency strain and the other of each pair was of the low efficiency strain.

The animals were matched in pairs according to body weight and were operated on under ether anesthesia with satisfactory surgical technic. The adrenal glands were removed from one animal through a lumbar incision and sutured to the surface of the ovaries of the other animal of the pair. Then the adrenal glands of the second animal were removed and likewise sutured to the ovaries of the first. All animals were given maintenance doses of cortin for ten days after the operation. A standard diet was fed which contained 1 per cent of potassium and 0.24 per cent of sodium. All animals were operated on and cared for by one of us who was unaware of the identity of the various animals until the time the grafts were removed for observation and study. All grafts, together with the ovaries, were removed surgically from those animals which survived four months, or 120 days. These animals were again given maintenance doses of cortin for ten days after removal of the grafts and their subsequent periods of survival were noted.

RESULTS. 1. *Homografts of the adrenal glands of rats of the high efficiency strain.* In the first series of experiments, three animals of the twenty-four which comprised this group developed symptoms of adrenal insufficiency and succumbed within fifteen days after conclusion of the first period of treatment with cortin. Histologic examination of these grafts disclosed that they had undergone complete degeneration. Twenty-one animals in this series gained in weight and remained entirely free from symptoms of adrenal insufficiency for 120 days, when their grafts were removed. Twenty animals developed symptoms of adrenal insufficiency and died within forty days after the second period of treatment with cortin had ceased; while one animal lived indefinitely, apparently in good

health. Histologic examination of the grafts which were removed from these animals disclosed that viable cortical tissue was present in every case. In the grafts from seven animals there were slight amounts of lymphocytic infiltration; while the grafts from fourteen were entirely free from lymphocytes or other evidences of antagonistic host reactions.

In the second series of experiments, only one animal of the group of eighteen developed symptoms of adrenal insufficiency and succumbed within twenty days after the postoperative treatment with cortin was withdrawn. Histologic examination of the grafts disclosed that they had degenerated completely. Seventeen animals of this series remained free from symptoms of adrenal insufficiency for the period of 120 days. The grafts were removed and all of the seventeen animals then developed symptoms of insufficiency and died within forty days after conclusion of the second period of administration of cortin. Histologic examination of all grafts likewise disclosed that viable cortical tissue was present in every case. In grafts of eight animals there were varying amounts of lymphocytic invasion but in grafts of nine of them no cellular infiltration occurred.

2. *Homeografts of the adrenal glands of rats of the low efficiency strain.* In the third series of experiments, three animals of the fourteen developed symptoms of adrenal insufficiency and succumbed within twenty-five days and one lived for eighty-five days after the first period of treatment with cortin had ended. Histologic examination disclosed that the grafts of these animals had completely degenerated. Ten animals of this group remained free from symptoms of insufficiency until removal of their grafts 120 days later. They all then developed symptoms of adrenal insufficiency and succumbed within forty days after treatment with cortin was withdrawn. Histologic examination disclosed viable cortical tissue in every case. The grafts from two of the ten animals gave evidence of some cellular invasion but those of the remaining eight were entirely free from host lymphocytes.

3. *Homeografts of the adrenal glands between animals of the high efficiency and low efficiency strains.* In the fourth series of experiments twenty-one of the twenty-six animals comprising this group developed symptoms of adrenal insufficiency and succumbed within thirty-five days after the first postoperative treatment with cortin was withdrawn. Three of them lived for fifty days and one lived for 100 days. Histologic examination of all of these grafts disclosed that they were completely degenerated. Only one animal of the twenty-six survived for 120 days. Its grafts were then removed, but the animal lived indefinitely and apparently in good health. Histologic examination of these grafts disclosed that they too were completely degenerated.

The results of these four series of experiments are graphically summarized in figure 1.

COMMENT. The importance of the genetic relationship between donor and host in the success or failure of these adrenal homeografts is clearly apparent from our study. Loeb and King have stressed the importance of this relationship in their study of thyroid homeografts and have shown that the degree of compatibility between host and graft depends on the degree of identity between the organismal differentials of the two animals. Organismal differentials are the biologic or chemical characteristics of organisms determined by their genetic constitutions. Loeb and King

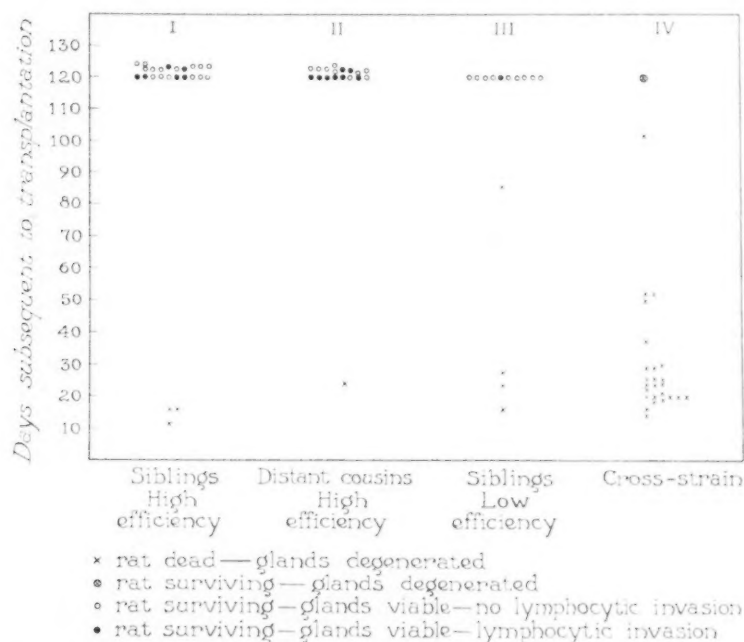


Fig. 1. Survival time of animals which received adrenal homeografts in the experiments described in the text.

have shown that increasing the distance of relationship between the donor and host increases the degree of fluid and cellular reaction against the graft. It has been suggested that the success or failure of homeografts among distant cousins may well be employed as an index of the degree of homogeneity in any given strain.

Of the twenty-eight pairs of animals which received homeografts within their respective strains only eight animals, or 14 per cent, failed to establish viable grafts. Some of the failures may have been owing to mechanical factors such as improperly placing the graft against the host tissue.

But the lymphocytic and giant cell reaction by the host against the graft demonstrated a biologic or a chemical incompatibility between the donor and the host. So that even in closely inbred strains, as in these two under experiment, complete biologic compatibility may not always exist. In our second experiment, wherein the glands of distant cousins within the high efficiency strain were exchanged, only one rat of the eighteen operated on failed to develop viable grafts; the results following grafting between distant cousins, therefore, appeared to be better than those obtained when glands were exchanged between sisters. We believe, however, this difference is owing to chance and not to the character of the crosses; the percentage difference in results is slight.

Homogeneity within each of the two strains was clearly demonstrated in our series of experiments, and the results confirm the opinion that grafting of an endocrine organ, such as the adrenal gland, may be an index of the degree of genetic identity of the animals within a given strain.

In each cross homeograft performed in the fourth experiment, whether the graft was from an animal of the low efficiency strain to one of the high efficiency strain, or vice versa, the host's cytologic reactions against the graft were sufficient in each instance to destroy it. The one animal which survived had degenerated grafts, just as all the others had, but an accessory adrenal gland maintained life. Burhoe (2) showed that our strains belonged to different blood groups. He showed that the serum of one strain was found to agglutinate the cells of the other strain. This difference in blood grouping is probably not a factor in our failure to secure viable cross strain grafts, for unpublished data (2) indicate that the blood grouping of donor and host does not influence the viability of transplants of certain other types of tissue.

Lymphocytic infiltration of a graft is ordinarily interpreted to indicate a biologic incompatibility between host and graft (5, 6), and in general the more distantly related the donor to the host the greater will be the lymphocytic reaction of the host against the graft. The most extreme reactions have been encountered in hosts which have been grafted with tissues of another species (5). In grafts removed from our animals we have found considerable variation in the degree of lymphocytic invasion. Even in many of the grafts which survived for four months large numbers of lymphocytes were present. In several instances the lymphocytic reaction was observed in viable grafts of but one animal of a given pair. Furthermore a lymphocytic reaction was sometimes observed in one grafted gland but not in the other gland of the same animal. These variations in the lymphocytic infiltration of the grafts are difficult to explain and must indicate that the presence of lymphocytes may not be owing, necessarily, to host-graft incompatibility.

Biologic incompatibility, manifested by a lymphocytic reaction against a graft, may not be present for some time after grafting. A functional

adrenal graft is really a regenerated adrenal gland. A gland, immediately on grafting, degenerates except for a small rim of glomerular zone and the peripheral capsule. Regeneration of the cortical tissue proceeds from the capsule and from this narrow zone of glomerular cells beneath it. Often we have observed that grafts removed from animals which had died in a state of insufficiency gave every sign of complete regeneration and yet were infiltrated with lymphocytes. Regeneration had proceeded in the normal manner; subsequently, certain products probably had been elaborated by the graft which called forth showers of lymphocytes in the host and destroyed the graft. Such instances indicate that incompatibility between host and graft may not appear until weeks after a graft apparently has become established. We selected the period of four months (120 days) following grafting as one sufficiently long to indicate whether or not these grafts were to survive. However, the occurrence of varying numbers of lymphocytes in some of our grafts, even at that time, has led us to feel that had we continued our observations for longer periods some of our viable grafts might have been destroyed.

SUMMARY

This report covers the results of a series of experiments in which homeo-grafts of the adrenal gland were made (1) within two closely inbred strains of rats and (2) between animals of these two inbred strains. Two widely separated strains of rats, each known to have been inbred for many generations were selected. Rats of one of these strains were of high efficiency in utilization of food and the other of low efficiency. The following series of experiments were made: 1. The adrenal glands were exchanged between twelve pairs of adult sisters of the high efficiency strain. 2. The adrenal glands were exchanged between nine pairs of adult distant cousins of the high efficiency strain. 3. The adrenal glands were exchanged between seven pairs of adult sisters of the low efficiency strain. 4. The adrenal glands were exchanged between thirteen rats of the high efficiency strain and thirteen rats of the low efficiency strain. In all exchanges the rats were matched as to age and body weight and the glands were grafted to the ovaries. Four months (120 days) was considered to be a sufficient interval of time following operation to determine the success or failure of a graft. All animals which lived four months were explored and the grafts, together with the ovaries, were removed. The survival time of each animal following removal of the grafts was noted. The following results were obtained.

1. Of the fifty-six animals grafted with adrenal glands taken from animals within their respective strain, forty-eight animals survived with viable cortical tissue. The grafts in eight rats degenerated completely and the animals died of adrenal insufficiency soon after grafting.

2. Of the twenty-six animals grafted with adrenal glands taken from

animals of the other strain, twenty-five animals died of adrenal insufficiency and the grafts were extensively infiltrated with lymphocytes and giant cells. One lived 120 days but was found to have accessory adrenal tissue. Its grafts had likewise degenerated.

3. Close similarity in the genetic constitution of donor and host is essential for the regeneration and function of homeografts of the adrenal gland.

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RELATIONSHIP OF THE CONDITIONED OLFACTORY-FORE- LEG RESPONSE TO THE MOTOR CENTERS OF THE BRAIN

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The purpose of this investigation is to determine by the aid of extirpations the effective area or areas of the brain concerned with eliciting the genuine conditioned olfactory-foreleg response, which is positive to cloves with shock and negative to asafetida without shock. The establishment of such a center or centers would appear to be the best starting point for a physiological analysis of the olfactory system.

A complete description of the procedure used in recording these tests was given in an earlier paper in which it was demonstrated that inhalation of clove oil, anise, asafetida, xylol and benzol evoked this conditioned response in dogs only over the olfactory nerves; while many other vapors, camphor, eucalyptus, ether, chloroform, phenol (sheep dip), acetic acid, etc., were effective over the trigeminal as well as the olfactory nerves. These experiments were conducted in rooms in which an electric motor and air pump were run continuously to provide ventilation and to make other noises inaudible. All dogs were blindfolded during the tests and a sufficient number of empty bottle controls were presented. The animals were all young and alert selected from the ordinary run of laboratory dogs.

Anatomy discloses at least two possible cerebral areas having direct or relayed connections with the motor nuclei of the spinal cord. In addition to the pyramidal and extra pyramidal centers there is a second cortical area in the cornu ammonis having in the hippocampus several rows of pyramidal cells which traverse the fornix to relay in the thalamus to lower motor neurons. These hippocampal cells synapse with many afferent olfactory fibers. Outside the cerebrum several effective thalamic nuclei are in communication with afferent olfactory fibers and the motor nuclei of the spinal cord. Thus far physiology has furnished no positive evidence why any of these three areas should not be considered as possible effective centers for olfactory conditioned reflexes.

Elimination of the hippocampi. To determine if the hippocampi are essential efferent centers for the olfactory conditioned reflex both fornices were transected at their exit from the hippocampi in 9 dogs. In some animals conditioning preceded, in others it followed the operation. Two

methods were used in cutting the fornices. The first, used in all the early experiments, consisted of inserting a small chisel (made from a heavy piece of steel wire) a little behind the sulcus ansatus in a transverse plane from the median saggital sinus laterally to the depth of a notch that had been filed on the chisel. The cutting edge of the chisel was then moved laterally to insure complete severance of the fornices. This procedure was repeated on the opposite side. The second method, used in

TABLE 1
Conditioned olfactory reflexes after fornices were severed

DOG NUMBER	1ST RESPONSE TO CLOVES, RIGHT LEG	CLOVE REFLEX ESTABLISHED, RIGHT LEG	1ST RESPONSE TO CLOVES, LEFT LEG	CLOVE REFLEX ESTABLISHED, LEFT LEG	CLOVE REFLEX TRANSFERRED, LEFT LEG TO RIGHT LEG	DIFFERENTIATION CLOVES AND ASA FETIDA	CONTROLS
1**	7th trial	8th on	9th trial, 1-7, R. leg = +	14th on	6th trial, 1-5, R. or both = +	Yes	0
2**	15th trial	19th on	6th trial, 1-5, R. leg = +	7th on	2d trial, 1, L. leg = +	Asa. all 0	0
3**	31st trial	32d on	1st trial, 2-4 = 0	5th on	2d trial, 1, L. leg = +	Asa. all 0	0
4*	3d trial	4th on	2d trial, 1st, R. leg = +	3d on	1st trial	Asa. all 0	0
5*	17th trial	18th on	56th trial, 1-25, R. leg = +	75th on	4th trial, 1-3 = 0	None	0
6*	138th trial	160th on 130 to 160 = +, general movements	26th trial, 1-11, R. leg	27th on 11-25 = +, general movements		Yes	0

* Fornices cut after first method.

** Fornices cut after second method.

all the later experiments, consisted of exposing only the left cerebrum for a short distance behind the sulcus ansatus. A spatula was passed between the hemispheres and the sensory area of the left hemisphere was retracted laterally to expose the corpus callosum. Since this commissure is very thin in the dog it required but a shallow thrust of a chisel to sever the left fornix and only a slightly longer oblique thrust to transect the right fornix.¹

The full damage of the lesion was obtained in each instance from a study

¹ The sodium amytal used for anesthesia was generously donated by the Eli Lilly Co.

of the following sections: 1, a series through the lesion stained with hematoxylin and eosin; 2, sections of both hippocampi prepared after Nissl and Marchi methods to show cell chromatolysis and medullated fiber degeneration; 3, Marchi sections through the hypothalamus to show fiber degeneration in both fornices; 4, Marchi sections through the pyramids for fiber degeneration.

Both fornices transected before conditioning was attempted. There were 6 dogs in which this lesion was made with little or no damage to other struc-

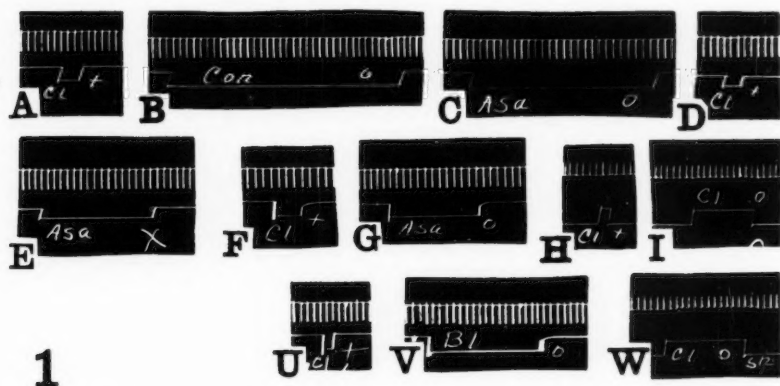


Fig. 1. Upper tracing, time in seconds; lower tracing, first break in line, signal for presenting vapor or control, second break, positive (+) or negative (-) response. A, positive clove, B, negative control and C, negative asafetida responses from right foreleg of dog which had both fornices sectioned; D and E, positive clove and much delayed asafetida responses from left foreleg of same dog as above. F and G, positive clove and negative asafetida responses from a fornix sectioned dog that had previously been conditioned to cloves. H and I, positive and negative clove records from the normal and paralyzed legs of a dog that had motor cortex removed from one side and both fornices severed. U and V, positive clove and negative control from right (paralyzed) leg after removal of the left motor cortex; W, negative clove record (327th) from right leg (same as U and V) after complete removal of the motor cortex.

tures except sensory cortex above and some commissural fibers. From a previous report it can be assumed that the average normal dog would record his first conditioned response some time during the first 25 trials.

A summary of the responses of these dogs appears in table 1.

In a typical dog (no. 1) an early positive clove response for the right foreleg appears in figure 1 A, while B and C are records of a negative asafetida and a control.² One of the positive clove and long delayed asafetida responses taken during the second series of tests when electrode was

² All failures to raise foreleg bearing the electrode during clove inhalation were punished by shock; while the asafetida tests were never followed by shock.

transferred to left leg appear in figure 1, D and E. Serial sections through the lesion of this dog reveals both fornices severed with no damage to the

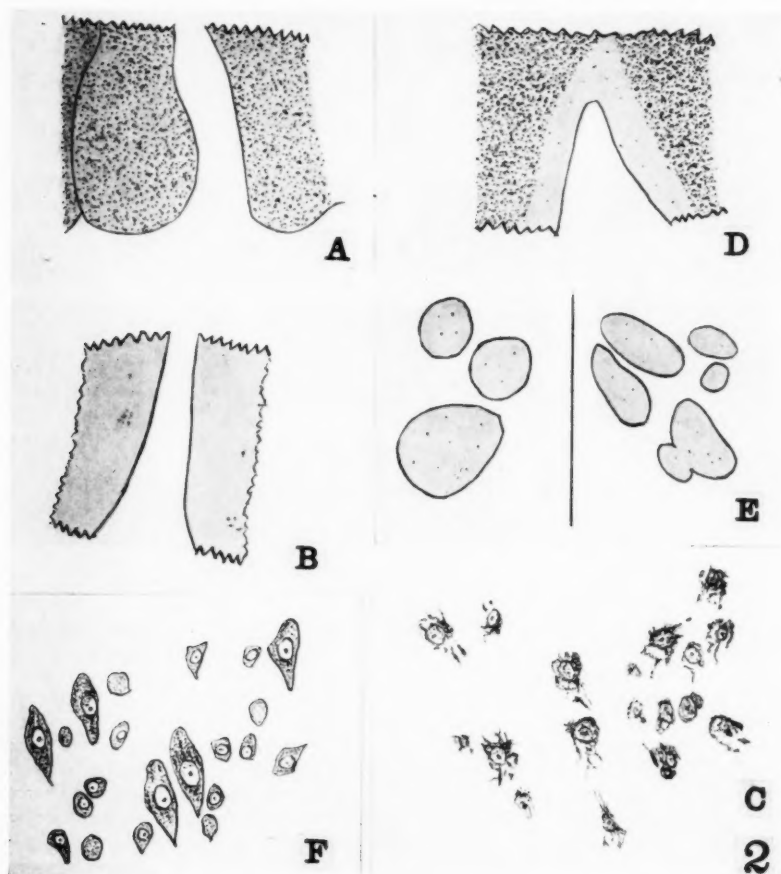


Fig. 2. A to C. from dog which had fornices transected at their exit from hippocampi; A, degeneration in fornices from Marchi section through hypothalamus; B, no degeneration in pyramids; C, complete chromatolysis of pyramidal cells from Nissl section through R. hippocampus. D to F, from dog which had motor cortex removed from both sides; D, degeneration in Marchi section through pyramids; E, no degeneration in fornices from Marchi section through hypothalamus; F, cells all normal in pyramidal layer R. hippocampus, Nissl section.

deeper thalamic structures. There was some injury to the adjacent corpus callosum and sensory cortex. That the fornices were severed without

damage to the cortico-spinal system is shown by the following control slides: 1. Marchi sections (fig. 2, A) through the hypothalamus of this dog directly in front of the mammillary bodies demonstrate complete degeneration of all efferent medullated hippocampial fibers in the fornices. Only the ventro-median portions of the fornices appear in this figure. 2. Nissl-stained sections through the hippocampi show an advanced stage of chromatolysis of all cells in the pyramidal layer. A small portion of this layer from the right hippocampus is portrayed in figure 2, C drawn by the aid of a camera-lucida. 3. A Marchi section through the median portion of the pyramids (fig. 2, B) discloses no more than the usual number of degenerated fibers found in any normal animal.

It is clear from table 1 that all 6 dogs acquired a positive foreleg response to cloves and a negative or much delayed response to asafetida.

TABLE 2
Conditioned smell reflexes

DOG NUMBER	BEFORE FORNICES CUT		AFTER FORNICES SEVERED					Controls
	1st response to cloves, right leg	Clove reflex established, right leg	1st response to cloves, right leg	Clove reflex established, right leg	1st response to cloves, left leg	Clove reflex established, left leg	Differentiation, cloves and asafetida	
7	9th, 3 sec.	10-25, 2-4 sec.	14th, 4 sec.	14th on 2-4 sec.	5th, 4 sec.	6th on 2-4 sec.	Asa. 0 or +10-15 sec.	0
8	23d, 4 sec.	24th on 2-4 sec.	1st, 5 sec.	2d on 1-3 sec.			Asa. 0	0
9	7th, 3 sec.	8th on 2-4 sec.	1st, 4 sec.	2d on 1-2 sec.	136th, 5 sec. 2-25+, R. leg	139th, on 2-4 sec.	Asa. 0 or +10-15 sec.	0

Dog 4 has broken all records to date with the fewness of tests required to establish the conditioned clove reflex. After learning the reflex this dog would respond correctly the first time the electrode was changed from one leg to the other. Table 1 also discloses that the only dog (6) to suffer any delay in olfactory conditioning through severance of the fornices was one in which the first operative procedure was used. This method is obviously far more destructive than the second method. It can be stated for dog 6 that Marchi sections revealed but few degenerated fibers in the pyramids and serial sections showed no apparent damage to the thalamus. There was some difference of behavior during a test. Dogs 1, 3, 4 and 5 simply turned their head from cloves, dog 2 growled, dog 6 showed rage symptoms and bit in the direction of the odor.

When blindfolded all of these dogs were able immediately to select a

paper packet containing meat from 3 paper packages of like size when placed in a pan or on the floor.

Conditioned to cloves before the fornices were transected. A brief summary of the conditioned responses before and after the fornices were severed appears in table 2. Of this group dog 7 had fornices cut by second method while the first method was used for dogs 8 and 9. Except for injury to some of the right cortico-spinal fibers in dog 9 and the usual damage to the sensory cortex and corpus callosum the control sections showed little or no damage to other structures.

One of the early responses of the right leg of dog 7 to cloves after the fornices were cut is shown in figure 1, F and a negative response to asafetida for 13 seconds appears in figure 1, G. Dog 9 whined with each presentation of cloves while dogs 7 and 8 simply turned their head in the opposite direction. When blindfolded these dogs were able instantly to select a paper package containing meat from 3 paper packages of like size.

Both fornices transected and cortico-spinal fibers of one side severed. Control sections and general behavior demonstrated that these lesions had occurred in two animals.

One of these dogs (no. 10) failed to raise the paralyzed (left) leg to 150 trials of cloves and the normal (right) leg to 65 trials of cloves. Dog 11 on the other hand responded to cloves with the normal leg (fig. 1, H) in 2 seconds during the 76th trial; while the paralyzed (right) leg yielded no genuine conditioned response to cloves for 185 trials. The last record is figure 1, I.

Dog 11 suggests that the normal leg of dog 10 would have responded with additional trials, but as to the effect of additional trials for the paralyzed leg of either dog, a guess would be untenable.

Elimination of the motor cortex. A little more than the excitable cortex, namely, an area bounded by the precruciate, longitudinal, ansate and coronal sulci and extending a short distance into the cephalic end of the coronal gyrus was removed to a depth of 9 to 10 mm. or to the level of the white matter in 5 dogs. In one dog the lesion was limited to the fore and hind leg, excitable areas of the posterior sigmoid gyri as determined by electrical stimulation before removal. A two stage operation was used, allowing a two week interval between removal of the two sides. The margins of each area were cut to the correct depth by a chisel possessing a depth gauge and the entire area was removed in one piece with a spatula. Autopsy disclosed little or no damage to neighboring structures. Marchi sections through the medulla and hypothalamus showed complete degeneration of all medullated fibers in both pyramids (see fig. 2, D for a portion of the pyramids from dog 12) and the fornices contained only the ordinary number of degenerated fibers found in every normal preparation (see fig. 2, E for portions of both fornices in dog 12). Nissl preparations revealed

only normal cells in the pyramidal layer of the hippocampi (fig. 2, F). These dogs exhibited the occasional slipping outward of the forelegs and backward of the hind legs together with the absence of the placing reflex which are the most characteristic permanent symptoms of dogs without motor cortex. When blindfolded they could instantly select a meat package from 3 paper packages.

Four of these dogs were conditioned on the paralyzed side after the unilateral lesion but no conditioning was attempted in two dogs until the motor cortex had been removed from both sides. Since this group of dogs became highly excitable during the later tests it was necessary to watch

TABLE 3

DOG NUMBER	AFTER MOTOR CORTEX WAS EXTIRPATED ON ONE SIDE		AFTER MOTOR CORTEX WAS EXTIRPATED ON BOTH SIDES		
	Number of trials to elicit 1st response with paralyzed leg	Number of trials to elicit 1st response with normal leg	Number of trials in which no true reflex appeared	Number of trials to evoke 1st generalized response	Behavior during the negative clove test
12	15*		327	197†	Turns head, whines louder, resp. inh.
13	77**	4	125††	None	Turns head and growls louder
14	152*	1	200	None	Turns head, whining stopped
15	97*	2	323	37	More excited, licks nose
16			305	91	Waves head, more excitable, whines
17			325	None	More quiet, stops whining, licks lips

* Differentiates between cloves and asafetida.

** Does not differentiate between cloves and asafetida.

† 25 inhalations of acetic acid (chiefly a trigeminal stimulus) were negative.

†† Dog died suddenly from unknown cause.

very closely the behavior of these animals at all times, to await quiet periods before making tests and to take many controls. A summary of some of the results from a multitude of tests made on this group of dogs appears in table 3.

It is apparent from table 3 that dog 12 acquired the clove-foreleg response (fig. 1, U) remarkably early in the paralyzed leg after unilateral extirpation of the motor cortex.³ Later dog 12 differentiated nicely by

³ This is the earliest conditioned response to cloves thus far obtained from a paralyzed leg after unilateral extirpation of the motor cortex. It usually required 70 to 150 trials. There is however considerable variation in the time of appearance of this reflex in normal dogs.

responding to cloves within 2 seconds and not responding to asafetida during 15 seconds. The controls (fig. 1, V) were all negative. The 327th negative clove response (fig. 1, W) was recorded 5 months after the second (right) motor cortex had been removed.

Dog 15 developed the so-called generalized conditioned reflex the best of any of these dogs. In this animal the usual response was a sliding of the left foot forward and a forward and upward movement of the right

TABLE 4

TEST	TIME OF INHALA- TION	BEHAVIOR DURING THE TEST	SHOCK	BEHAVIOR DURING THE INTERVAL BETWEEN TESTS
	<i>seconds</i>			
Cl. 251*	7	Quiet, licked nose	Yes	Quiet, licked nose
Cl. 252	7	Quiet, licked nose	Yes	Quiet, licked nose
Cl. 253	7	Quiet, licked nose	Yes	Quiet, licked nose
Cl. 254	7	Quiet, licked nose	Yes	Quiet, licked nose
Cl. 255	7	Quiet, licked nose	Yes	Quiet, licked nose
Cl. 256	7	At 4 sec. slid L. foreleg forward, then slight forward movement R. foreleg	Yes	Head movements, howled louder
Cl. 257	7	At 5 sec. slid L. foreleg forward, then R. foreleg forward and up	Yes	Head and body movements
Cl. 258	7	At 3 sec. slid L. foreleg forward followed by 3 elevations R. foreleg	No	Violent movements of head, R. foreleg elevated many times
Cl. 259	7	In 2 sec. slid L. foreleg forward, then R., L., and R. forelegs were raised	No	R. foreleg raised several times
Control	2	R. foreleg raised	No	R. foreleg raised
Control	10	After 3 sec. R. foreleg raised 3 times	No	R. foreleg raised many times
Asafetida	7	Raised R., L., R., and L. forelegs in order listed	No	Dog went into a tantrum of escape movements and could not be used for additional test

* Except for howling the dog was quiet before the tests.

(one bearing the electrode) foot. There were more movements of the right leg than the left. A summary of the results of one series of tests from dog 15 has been introduced in table 4 to show the direct relationship between the state of excitability of the dog and the presence or absence of the so-called generalized response. Other tables compiled from dog 15 showed same relationship but in reverse order to table 4. The dog started off with the generalized response when placed in harness but when ir-

ritability diminished the responses ceased both during and between the clove tests.

Dogs 13 and 14 were less excitable and showed no evidence of the so-called generalized response. Dog 17 in which the lesion was confined to the excitable area affecting movements of the fore and hind legs was of especial interest in that while this response never occurred during 325 clove trials followed by shock, a similar behavior appeared during the interval between tests. This behavior consisted of a few or a multitude of escape movements, preceded or intermingled with one or more elevations of the foreleg bearing the electrode. It should be noted that the above mentioned behavior never occurred until several tests had been made, but having appeared in an experiment, it would happen during many of the remaining intervals between tests. In dog 17 it was then possible, in spite of punishment, for the odor of cloves to change a short period that included escape movements and howling into one of apathy.

There was no sign of a negative generalized conditioned response to asafetida in any of these dogs.

Fornix transected on one side together with severance of the corticospinal fibers of the same side. Control sections demonstrated that this was accomplished in 2 dogs in which an attempt had been made to sever both fornices after the first method. Both dogs were conditioned positively to cloves and negatively to asafetida before the lesion. After the lesion they showed no interruption to the reflex on the ipsilateral (normal) side, but about the same number of trials were required to reestablish the clove response for the contralateral (paralyzed) foreleg as was necessary after unilateral extirpation of the motor cortex alone. The asafetida responses continued negative.

DISCUSSION. Concerning the rôle of the cerebrum in olfaction, Munk, Ferrier and Luciani claim that bilateral lesions which involve the ventral portions of the frontal lobes with some damage to the hippocampi resulted in the total loss of smell. Zavadsky reports that complete removal of the pyriform cortex together with some of the adjacent hippocampus did not cause complete loss of olfactory function in dogs. Experiments were cited in which he was able to reestablish a conditioned flow of saliva to presentation of a meat packet and also a preestablished conditioned response to camphor (a trigeminal as well as olfactory stimulant). Henschen records that complete unilateral destruction of the uncus, gyrus hippocampus and cornu ammonis does not result in any marked disturbance to smell. Elsberg et al. have stressed the value of olfactory fatigue in olfactory localization. Swann states that very large portions of the cerebral hemispheres and some of the thalamus can be removed in rats without effecting olfactory discrimination. The choice of "sheep dip" (a trigeminal as well as olfactory stimulant) for one of his vapors would seem to have been a most unfortunate selection.

Poltyrev and Zeliony reported at the Boston Physiological Congress of conditioning a supposedly decorticated dog to the sound of a hunting whistle. Their dog was said to raise his left leg and throw body backward to avoid an electric shock. Culler and Mettler have described a sort of generalized or diffuse auditory and optic conditioning for a decorticated dog. These responses suggested only struggles to escape.

It is clear from the foregoing tests that elimination of the efferent impulses from the hippocampi by way of the fornices caused no delay in the time required for learning a conditioned olfactory-foreleg response or any special interruption of this reflex if preestablished. Excluding the fornices in no way interfered with acquisition of a negative olfactory conditioned response or the ability to transfer an olfactory conditioned reflex from one foreleg to the other. On the other hand bilateral extirpation of the excitable cortex of the sigmoid gyrus not only prevented the establishment of an olfactory conditioned response of a foreleg but abolished a preestablished one. It may however permit of a sort of generalized conditioned behavior comparable to the diffuse auditory and optic conditioning that Culler and Mettler noted for a decorticated dog. Unilateral extirpation of the excitable cortex greatly delayed olfactory conditioning of the contralateral foreleg, but when once learned it could be transferred as quickly from the normal to the paralyzed leg as reversely and the reflex was fully as perfect and stable for the paralyzed leg.

It seldom happened in dogs having both fornices severed that any of the early clove tests, which failed to elicit a true conditioned foreleg response, evoked a generalized response of any kind. During the short process of learning the conditioned clove-foreleg response these dogs would simply turn their heads from the odor, sniff and in some instances whine. They rarely became excitable and as soon as the conditioned response was established they seemed to enjoy the procedure.

The so-called generalized response or behavior noted for half of the dogs without motor cortex did not occur until many negative responses to cloves with shock had been recorded. Simultaneously with the appearance of this response these dogs had one or more periods of excitement and one or more periods of apathy during every experiment. During the quiet period they rarely exhibited the so-called generalized response at the time of the test or between tests; while during an excitement period, which may occur when dog is first placed in harness or at any time during the experiment, this response or behavior may appear once or twice during each of several successive clove tests, during the controls and during the intervals between these tests. The response varied considerably even in the same animal. It usually consisted of isolated or mixed movements of all legs with the leg bearing the electrode being lifted most often. This is only natural since this leg is constantly being shocked and is more irritable.

On rare occasions, directly before or after an excitement period, 2 dogs which had been quiet for a short time directly before a clove test, raised the foreleg bearing the electrode during the test. Such a response if considered by itself might be taken for a conditioned olfactory-foreleg response, but when considered with the experiment as a whole, probably signifies only the first or last manifestation of an excitement period.

These periods of excitement in the motor cortex deleted dogs presumably originate from disagreeable memory associations for the whole procedure. The first associations start when the dog is being conducted from his quarters to the laboratory (indicated by a holding back which is in marked contrast to the fast pace of his return). These associations are reinforced by additional memories suggested by stimuli received when placed in the harness and the tests which soon follow.

There should be no confusion between the true foreleg response which takes place once and only once during clove inhalation in normal dogs and the so-called generalized response or behavior which takes place at any time during a period of excitement in dogs without motor cortex. The problem of the future is to determine the effects of extirpating certain areas that supply the motor cortex.

The only explanation that suggests itself concerning the absence of all leg movements at the time of the clove tests during the excitement periods in dog 17 (motor cortex deleted) is that the sensory and association side for a conditioned response is concentrating for action, but is prevented from so doing by the inability of the motor side of the reflex to function. The terrific shock punishment that this dog received during 325 clove tests certainly forbids calling it a negative conditioned olfactory response.

SUMMARY AND CONCLUSIONS

Transection of both fornices at exit from hippocampi caused no delay in establishment of a positive conditioned olfactory-foreleg response to cloves or a negative response to asafetida, nor any additional trials to transfer either reflex to the opposite foreleg or any interruption of these reflexes if acquired before the fornices were severed. It also in no way interfered with olfactory selection of a meat packet from paper packages of like size and texture.

Unilateral extirpation of the sigmoidal (excitable) cortex produced no delay in learning positive clove and negative asafetida conditioned responses for the homolateral (normal) leg, but greatly delayed conditioning the contralateral (paralyzed) leg. When once established these reflexes could be transferred as rapidly from the normal to the paralyzed leg as reversely and the reflexes were fully as stable for the paralyzed leg.

Bilateral extirpation of the excitable cortex prohibited acquisition of a true olfactory conditioned reflex for one leg or any conditioned differentia-

tion between cloves and asafetida. The relationship between a so-called generalized conditioned response and certain periods of excitement which developed simultaneously in half of the dogs without motor cortex is fully considered in the discussion.

Severance of one fornix and the cortico-spinal fibers of the same side caused no more delay in olfactory conditioning the paralyzed leg than sectioning the cortico-spinal fibers alone.

The conclusion seems warranted that the integrity of the excitable area of the sigmoid cortex of one hemisphere is essential for the establishment of a constant specific olfactory conditioned response consisting of raising one foreleg to avoid an electric shock or a continuation of the reflex if acquired before the lesion. This does not exclude the thalamus as a center for a low order of olfactory conditioning.

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STUDIES OF THE ENERGY OF METABOLISM OF NORMAL INDIVIDUALS

THE INTERINDIVIDUAL AND INTRAINDIVIDUAL VARIABILITY OF BASAL METABOLISM

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The inquiry reported here is sequential to our consideration of the subject of an adequate formulary basis for the standardization of basal metabolism. In considering alternative formulas for standardizing basal metabolism, one deals more or less automatically with the deviation of predicted from observed values. In a previous paper (1) we pointed out that a formula which predicts metabolism from sex, age, stature and weight, cannot predict closer in a representative series of such predictions than what is the normal variability of metabolism in a group of individuals of the same sex, age, stature and weight. This is the variability of total metabolism for fixed sex, age, stature and weight. To measure it directly would require the collection of a large number of individuals of the same sex, age and identical stature and weight, a manifest impracticability.

One may, however, calculate an average value of it indirectly, with sufficient accuracy for most purposes, if one has at hand a formula for estimating the mean metabolism of individuals of any given age, stature, weight, and for each sex. The variability of total metabolism for constant sex, age, stature and weight then becomes measurable as the variability of observed metabolism from the mean metabolism predicted by the formula. Using our own data this variability has been estimated from linear regression formulas, and from the "surface area" formula of DuBois in conjunction with The Mayo Foundation Standard for Normal Metabolism. Using the data of Harris and Benedict (2) it has been similarly estimated, using their linear formulas as well as a surface area standard appropriate to their data. The results have been given in a previous paper (tables 1 and 3, (1)), and since we shall presently proceed to a different formulation of these variabilities, we need only note here summarily that the average values of the variability of total metabolism between individuals of the same age, stature and weight, measured as standard deviation in calories

per hour, are as follows: for males aged 12-19, 4.8, aged 20 and over, 4.6; for females aged 12-19, 4.3, aged 20 and over, 3.9.

We have previously (1) argued that if standard metabolism is to be predicted from, or "corrected for," age, weight, and stature, the method of using the height-weight surface area formula of DuBois, and expressing metabolism as "calories per square meter," has certain heuristic advantages over a linear prediction formula in age, stature, and weight.¹ In accordance with this viewpoint we shall now take up our definitive consideration of the variability of metabolism in terms, not of total calories per hour for fixed stature and weight, but of calories per hour per unit area of body surface, the latter estimated by the DuBois formula in stature and weight.

The variability of metabolism has been studied in various terms by a number of previous workers. The first critical and methodical work is probably that of Benedict (4) and of Harris and Benedict (5), and other workers have contributed sporadically to the subject since. In this article our main task will be to present the results of calculations from our own data, and we shall not attempt to survey exhaustively the contributions of previous workers. Other authors will be cited only in so far as we have found that their quantitative results are comparable with ours and serve either to confirm or contradict our own.

We will study: 1, the interindividual variability, i.e., the variability among different individuals of the same sex and age a , in its totality, and b , as for the mean metabolism of such individuals; and 2, the intraindividual variability, i.e., the variability of determinations made on the same person, a , from day to day, and b , intradaily.

Interindividual variability. Our data for the examination of the total interindividual variability comprise single observations of the basal metabolism of 639 male and 828 female subjects used for the erection of The Mayo Foundation Standards of Basal Metabolism. The details of the principles used in the selection of the subjects and the methods of measurement are given in a former publication (6). The deviation of the observed metabolism of each individual, expressed as calories per square meter per hour, from the mean given by The Mayo Foundation Standard for the individual's sex and age was determined. To obtain an average picture, the data were assembled by age for each sex in two groups: 1, those under 20 years of age, and 2, those 20 years and over. In each of the resulting four groups the deviations of observed from standard mean metabolism

¹ Since this paper was written, Talbot, Wilson, and Worcester have published an article (3) advancing the use for children of two standards of metabolism, one based on weight and the other on stature, each to be adjusted for age. Their study contains a meticulous statistical analysis, but none of their results have modified our previous opinion that a standard based on "calories per sq. meter" (DuBois) is the most practical and the most efficient, if age, stature and weight are the basis of the standardization.

were assembled into a frequency distribution, the standard deviation was determined, and the form of distribution studied. The results are shown in figure 1.

For each of the four groups the distribution was well in accord with the gaussian curve.² This is evidenced by a comparison of the percentage frequency that would be expected in a distribution that followed the gaussian curve and the frequencies observed, and also by the application of the chi-square test for goodness of fit.³ As shown in figure 1, we found as the standard deviation for males under 20 years of age, 3.14 calories per square meter per hour (6.5 per cent of mean), and for those 20 years and over, 2.58 (6.7 per cent of mean). For females the standard deviation in the group under 20 years was 2.98 calories per square meter per hour (6.8 per cent of mean), and for those 20 years and over, 2.42 (6.9 per cent of mean). The variability measured in units of calories per square meter is greater for males than for females, and greater for younger than for the older ages. If the relative variability is considered, i.e., the standard deviation expressed as a per cent of the mean, the variability is somewhat greater for females than for males, and greater for older than for younger persons.

To compare our results with those for another large series of normal subjects, we examined the data of Harris and Benedict, given in their *Biomet-*

² Also called the "normal" curve, the "curve of error," etc. If names are to be used in chronologic order of discovery, it should be called the "DeMoivre-Laplace-gaussian curve": vide K. Pearson *Biometrika* 16: 402, 1924.

³ The frequencies are compared in the figures according to the per cent of cases beyond specific deviations, because one usually wishes to know the fraction of cases that fall beyond a given value. For the chi-square test the frequencies *within* intervals of deviation are the ones compared. This test is in great vogue among statisticians. A value of P less than 0.05 is considered to indicate a "poor fit." In the opinion of one of us (Berkson), the test may, for a variety of reasons, be frequently unsatisfactory and misleading in practice. One reason is that when the sample is very large numerically, even relatively small differences, especially toward the extremes of the distribution, yield a low value for P and will prove "significant." This is correct so far as the mathematics of the test is concerned, since even small differences from expected frequencies are unlikely in random sampling if a large sample is used. But these are differences which in practice are very frequently not "significant," in the sense that no important theoretical or practical meaning can be associated with them which would justify discarding the curve. Another point is that, if the sample is in very good accord with the curve, a very high value of P will result (two such cases are illustrated in figs. 1 and 3). According to Fisher this is to be interpreted exactly as would be a very small value of P (*Statistical Methods for Research Workers*, 4th ed., p. 83). Again, as a matter of mathematical probability this is correct since a high P indicates a sample as improbable as does an equivalently low P, and there are circumstances which would make these values equivalent in practice also. But in applications such as are made in this paper, to consider them equivalent would be absurd. The fact is that theorems in probability arrived at deductively from *a priori* hypotheses do not, in themselves, answer the question that is pertinent in practice when we wish to know whether a curve is a "good fit."

ric Analysis of Basal Metabolism (2). As a standard for mean metabolism per square meter to be used at various ages, appropriate to their data, we used the linear regression equations in these variables given by them (page 115) for their series of 136 males and 103 females. We confined our-

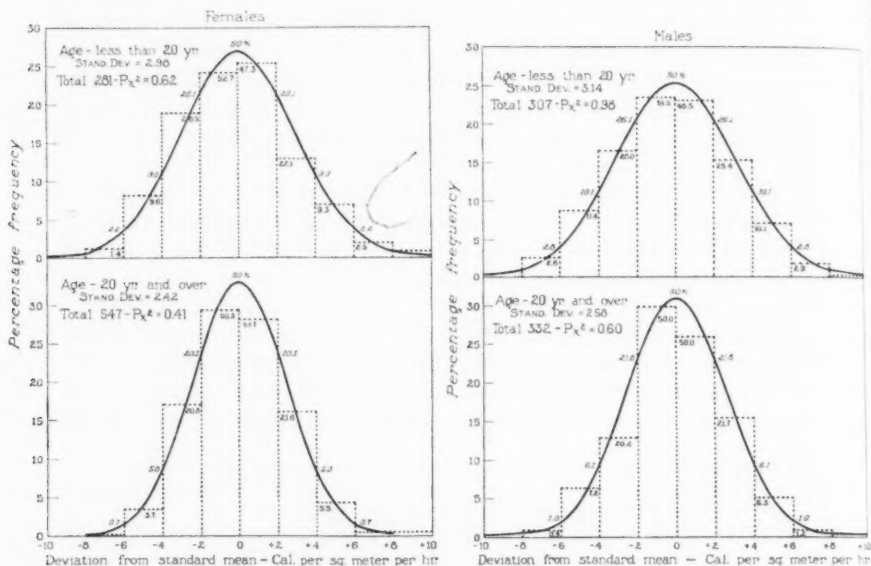


Fig. 1. Interindividual variability, total. Distribution of deviations from standard mean metabolism of individuals comprising the normal series of The Mayo Foundation (639 males, 828 females) compared with the "normal" or gaussian curve. The deviation of each individual was measured from the mean given by The Mayo Foundation Standard for that individual's age and sex. These deviations are grouped in the figure for males and females separately, and for ages less than 20 years and for 20 years and over. The gaussian curve was fitted by using as standard deviation the value of the standard deviation of the observed group. The P_{χ^2} in each figure gives the P corresponding to the chi-square test for goodness of fit. The number of "degrees of freedom" used for the test was 2 less than the number of frequency classes, since the observed total number and standard deviation were used for the fitted curves.

The percentages on the ordinate scale give the frequency for each 2 calories of deviation. The histogram in broken line corresponds to the observed values, the continuous line to the gaussian curve. The percentages printed in inclined numerals above the smooth curve give the theoretical expectation of deviations beyond the point indicated, and for comparison below these in upright figures are the corresponding observed percentage frequencies. For instance, in the figure for males less than 20 years old, the gaussian curve predicts that 26.1 per cent will have deviations of 2 calories or more above standard value; the observed frequency was 25.4 per cent. Similarly the gaussian curve predicts 10.1 per cent for deviations of 4 calories or more below the standard, the observed value being 11.4 per cent.

selves to the individuals 20 years and over who comprised all but a few of their series, and calculated the standard deviation from the mean metabolism given by their formulas. For males the standard deviation was 2.45 calories per square meter per hour (6.4 per cent of mean), and for the females 2.72 (7.7 per cent of the mean). These results are in general quantitative agreement with those obtained from our own data. However, the absolute variability is greater for females than males, which we did not find to be the case, and the relative variability is greater in females than in males by an amount larger than was true for our series. It is to be noted, however, that the observations recorded by Harris and Benedict are not in general *individual* observations, but the *means* of numerous observations made on the same individuals. It is a statistical fact that the variability of means will be less than the variability of the individual observations, and will be inversely proportional to the square-root of the number of observations used in the calculation of the means. Hence the differences of the findings in ours and Harris and Benedict's data might be accounted for if the number of observations for their recorded data of the males was greater than that for the females.⁴ To test this hypothesis we calculated the standard deviation for a supplementary series of 31 males given by them (p. 234) and which were apparently based on individual or few observations. The standard deviation for this series, calculated in the same manner as for their 136 males, was 2.83 calories per square meter per hour (7.3 per cent of mean). If the findings from this series are taken to represent males rather than those from their series of 136, the relation between the variability of females and males is nearer our findings, the absolute variability being larger for males but the relative variability larger for females.

Intraindividual variability. The interindividual variability just discussed, i.e., the fluctuation of the values of the observed metabolism (calories per square meter per hour) among individuals of the same sex and age, may be considered to be composed of a number of elements of variability each of which may be of separate interest. If for a particular individual repeated determinations of metabolism are made on successive days according to the usual procedures, these determinations will show fluctuations. If the period covered is not long, we may assume that these fluctuations take place around a more or less stable mean, characteristic of that individual at that time. These means around which the successive daily observations fluctuate, themselves vary from individual to individual. This may be assumed with reasonable assurance, even if the individuals are "controlled" for sex, age, stature, and weight, on the basis of the general observation that individuals are not identical one with

⁴ That this is in fact so is indicated by the number of observations in their tables C and D.

another in respect of any biological character. What we have measured as "total interindividual variability" is the resultant, then, of the variability of the means of the different subjects and of the variability of separate determinations on the same subjects. The last we shall refer to as "intraindividual variability." These variabilities measured as standard deviations are related as follows:

$$\sigma_t^2 = \sigma_i^2 + \sigma_{\bar{i}}^2 \quad (1)$$

where σ_t is the total interindividual variability, σ_i the intraindividual variability, and $\sigma_{\bar{i}}$ the interindividual variability of the individual means. We have already determined the value of σ_t . If we determine σ_i , we can calculate $\sigma_{\bar{i}}$ from equation (1) which would require for direct measurement the establishment of each individual's mean by an indefinitely large number of observations. The intraindividual variability (σ_i) can be determined if we have available numerous successive observations, each made under standard conditions for the same individual, and this for numerous individuals. Our data in point consisted of 10 females, aged 18 to 53 years, and 23 males, aged 18 to 40 years, on each of whom we made from 7 to 228 consecutive morning observations. In order to avoid the effect of possible long-time changes, we limited ourselves for purposes of calculating the intraindividual variability to a maximum of 15 consecutive determinations, taken over not longer than three months' interval for any particular individual. The standard deviation for the morning observations of each subject about the observed mean for that subject were calculated. To get a composite picture for each sex the deviations of each observation from the subject's mean were gathered into a single distribution and the standard deviation corresponding to the combined groups was calculated.⁵ The results are shown in figure 2. For males the standard deviation for the whole group was 1.33 calories per square meter per hour (3.5 per cent of the mean), and for females 1.61 calories per square meter per hour (4.7 per cent of the mean). The intraindividual variability in absolute terms, it is seen, was found to be greater for females than for males, the reverse relation from what we found for the interindividual

⁵ In estimating this value for the combined group the sum of the squares of the deviations was divided by the number of observations minus the number of individuals, since the latter is the number of observed means utilized. The same method was used wherever in this paper we combined data for different individuals from the observations of other authors. This will give the best estimate of the standard deviation of the population for which the observations are to be considered a sample. However, in the corresponding illustrative figures 1, 2 and 5, for which curves were fitted to be compared with the observations, the standard deviations used for the fitting were calculated by dividing by the total number of observations, since this gives the standard deviation of the particular sample which is depicted in the diagram.

variability. This greater variability of the females individually may be explicable as due to the disturbing effect of menstruation on metabolism.

The distribution of the deviations, as shown in figure 2, is in our opinion in reasonably good accord with the gaussian curve, though the chi-square test for the males yielded a value for P of only 0.04, which would be considered in routine statistical practice not a "good fit." The distribution is, however, quite symmetrical, and the discrepancies from the gaussian curve are erratic rather than systematic, so that we feel that the gaussian

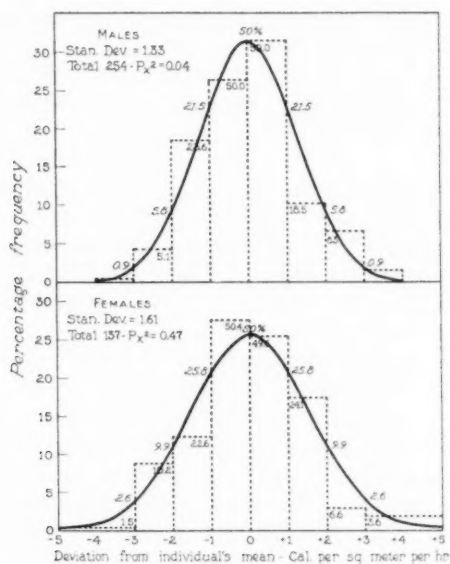


Fig. 2. Intraindividual variability: Distributions of deviations from mean metabolism of the individual. Average picture for numerous individuals. The standard deviation in the figure is the best estimate for the population of which the observations are to be considered samples; for fitting the curves the standard deviations of the samples were used. The explanatory notes in the legend of figure 1 apply here.

curve gives a good description of the form of distribution of the intraindividual deviations from the mean metabolism. It is not to be understood that, because we combined all the individuals in each sex, we imply that the intraindividual variability is the same for all ages and does not itself vary from person to person. On the contrary there is convincing evidence that the variability depends on what may be called "nervous stability" as well as on training for metabolism tests. We are concerned here, however, only in getting the general picture of the situation. In figure 3 is

shown the distribution of deviations from mean metabolism for two individuals separately, one male for whom there were 61 observations and one female for whom there were 190 observations.

Harris and Benedict, in a study of the variation of statistical constants in man (5), gave the calculated standard deviation in calories per square meter for each of 11 male subjects. From these we selected the individuals for whom there were not more than 30 successive observations, and calculated the variability for the combined group. The standard deviation in calories per square meter per hour was 1.43 (3.9 per cent of mean). This is somewhat greater than we found for our series of males.

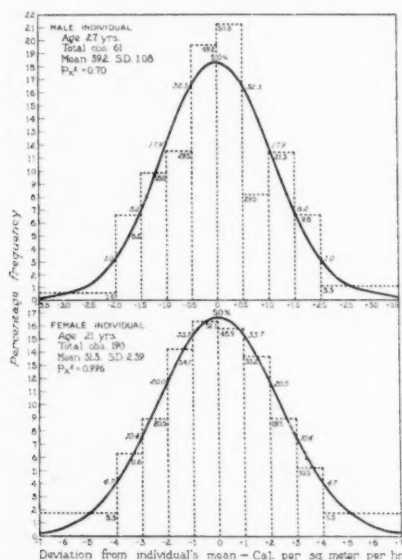


Fig. 3. Intraindividual variability: Distribution of deviations from mean metabolism. Two individuals; upper, male; lower, female. The explanatory notes in the legend of figure 1 apply here.

Blunt and Dye, in a study (7) of the basal metabolism of normal women, gave the successive daily observations over a period of a month for each of 17 subjects in calories per square meter. This enabled us to calculate the intraindividual variability, and for the entire group the standard deviation was 1.55 calories per square meter per hour (4.5 per cent of mean). Our findings for the series of females are very close to these.

Wishart (8) studied the intraindividual variability of metabolism in 5 subjects, 3 of whom were under continuous normal diet, one of them being a female and the other two males. He gave the relative variabilities measured as coefficient of variation; for the female it was 5.2 per cent, and the

average for the two males was 4.9 per cent. These variabilities are somewhat larger than we found in our own data, and also for the data of other authors that we have tabulated. This is probably attributable to the fact that Wishart's observations extended over a considerably longer period than the ones included in our own data; also the observations in the case of one of the males were not made under basal conditions.

Griffith and others (9) studied the metabolism of 2 men and 3 women continuously for an extended period and gave the standard deviation of the successive daily determinations. For the 2 men combined, the standard deviation was 1.18 calories per square meter (3.5 per cent of mean) and for the 3 women 1.34 (4.0 per cent of mean). The females showed a larger variability than the males, which our findings confirm. The values obtained by these investigators were somewhat smaller than in our results in spite of the fact that their observations extended over a period of two years.

Rubenstein, in a study (10) of the changes of body temperature and metabolism during the menstrual cycle, gave calculations of the standard deviation, in calories per hour, of repeated daily determinations of metabolism for 10 women. In a personal communication he supplied us with the surface area (DuBois) for each of these individuals, which enabled us to express the variabilities as calories per square meter per hour. For the 10 women taken together, the standard deviation was 1.62 calories per square meter per hour (4.9 per cent of the mean). Our findings for adult females are in close agreement with these.

Benedict and Carpenter (11) have published detailed observations of oxygen consumption under basal conditions for 3 men who were followed continuously during a part of the day and for many successive days, and Benedict published observations (12) on himself for successive days over an extended period. These enabled us to calculate the corresponding intra-individual variabilities. We selected from the observations on the 3 men in the Benedict and Carpenter series the initial observation in the morning for the first available fifteen successive days, so as to correspond with the selection of our own data. The standard deviation for the 3 men together in calories per square meter per hour was 1.65 (4.2 per cent of the mean). This is somewhat larger than what we found for our adult male group. Benedict's daily observations on himself are for two continuous periods of almost a month each. During this long period, except for a special interval of four days, the metabolism was remarkably constant, the standard deviation in calories per square meter being only 0.73 (2.2 per cent of the mean). We have encountered no such small value for so long a period in any other subject and attribute it to the unique training of this veteran investigator of metabolism. It is a notable example of the effect of training on the intraindividual variability.

Having now an estimate of the total variability (σ_t) and the intrain-

dividual variability (σ_i), we are in a position to evaluate the variability of the mean metabolism between different individuals from equation (1). Since the intraindividual variability has been calculated for adults, we will use the adult figures for the total interindividual variability. The calculated figures from our own data will be the ones utilized for this purpose. We have then for males $\sigma_t = 2.58$ calories per square meter per hour, $\sigma_i = 1.33$, and we obtain for σ_t 2.21 (5.8 per cent of mean). For the females $\sigma_t = 2.42$, $\sigma_i = 1.61$, and we obtain for σ_t 1.81 (5.2 per cent of the mean). Here we have an interesting result, for it appears that both the absolute and relative variability are greater in *males* than in females. While the differences are not large we believe they are representative.

We have here the conclusion, therefore, that if the characteristic (mean) metabolism of the individuals of the species is considered, adult males are more variable than females. Repeated determinations for individual females are, however, more variable than for males, perhaps as a result of the disturbing influence of the cyclic ovarian function. Therefore, when single determinations for many individuals are used to measure the variability, the total gives a larger net variability for females. It is as though we tried to compare the variability of stature in males and females under circumstances in which the measurement of height in the female was relatively unstable because of posture.

Intradaily variability. What we have discussed up to this point concerned calculations made with single observations, each taken in the morning under the usual standard post-absorptive conditions. We will now take up the consideration of successive observations, begun in the morning, as usual, but continued for a definite number of observations during the same day. Our data for this investigation of intradaily variability consisted of 19 adults (18 males and 1 female), for each of whom we had observations on from 3 to 196 days and for which there were on each day from 2 to 9 observations. We examined also the intradaily part of Benedict and Carpenter's data on 3 men, already cited (11), and also some published observations on Hitchcock (13).

We must first take up the possibility of a systematic trend of the observations as we proceed from the first to later observations during the day. Observations made progressively after the first one cannot be assumed to be homogeneous with earlier ones, since for each successive observation there has been a longer interval since the last meal, the period of experiment has been longer, and in general the conditions which might affect nervous stability have altered. In order to examine the daily data for a systematic trend, therefore, each observation was expressed as a deviation in calories per square meter from the mean for that day; that is, the mean value for the day was the zero base line from which each observation was measured. This was done because we are here interested

in tracing any regression *within* the day, and the average values of the different days, which will not necessarily be the same, are to be considered on the same level. All the first observations of the day measured in this way were averaged, also the second observations, third observations, etc., for as many as were available. The results are shown in figure 4 both for our own data and for those of Benedict and Carpenter. There is general agreement between the picture presented by the assembly from our data and that from those of Benedict and Carpenter. For the first 2 or 3 successive observations (which will mean on the average until about 9:30

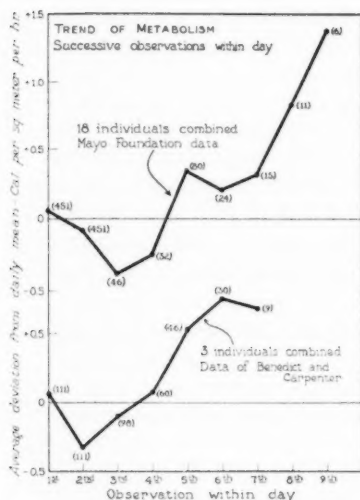


Fig. 4

Fig. 4. Trend of metabolism, successive observations within day: The numerals in parenthesis give the number of observations.

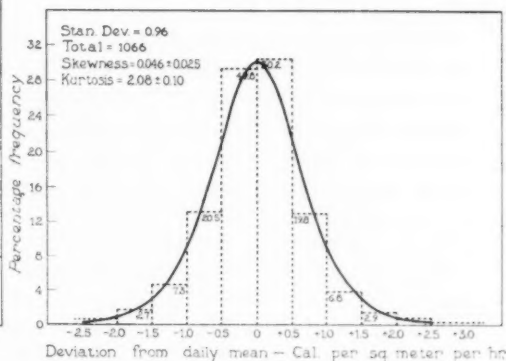


Fig. 5

Fig. 5. Intraindividual, intradaily, variability: Distribution of deviations from observed daily mean. The histogram in broken line represents the observed frequencies. The smoothed curve is a Pearson, type VII.

a.m.) there is a decrease in the metabolism amounting on the average to about 0.4 calorie per square meter. Then there is a rapid and continuous rise to above the initial value, so that by the 7th observation (about 12 noon), the mean increment of metabolism is positive and about 0.4 calorie per square meter. We may speculate that the initial lowering is correlated with an increasing adjustment to basal conditions, and that, as time passes, restlessness begins to be effective in raising the metabolism.

The cycle of changes in metabolism in continuous observations like those just described, that is, first a lowering and then an increase, can be

made apparent only if the averages of a large number of observations, which eliminate the fluctuating variations, are observed. For any particular subject on any particular day, generally the trend will be masked by what will appear as erratic fluctuations. The total variability within the day can be studied as we did the distribution of the variations of the interindividual and intraindividual variability, and this is done in figure 5. All the deviations from the daily means are gathered into a single distribution, to give an average picture and to estimate the value of the standard deviation for the entire group. The standard deviation for the distribution of intradaily variations was found to be 0.96 calorie per square meter per hour (2.7 per cent of the mean).⁶ In this instance, in contrast with interindividual and intraindividual variations from the means previously studied, the form of distribution was not in accord with the gaussian curve. The skewness was practically zero, that is, the distribution of deviations was quite symmetrical, the plus and minus deviations being equally frequent; but the distribution is leptokurtic when compared with the gaussian curve, which is to say there are relatively more deviations of small magnitude than the gaussian curve would anticipate. A type VII Pearson curve was fitted to the distribution, and this is given in the figure. Even this shows systematic departures from the observed frequencies of deviations from the daily means. In spite of this, for practical purposes we feel that the gaussian curve gives a satisfactory estimate of the deviations to be expected from the daily means. The lack of concordance of the assembled distribution with the gaussian function may be attributable to the fact that the number of observations within each day

⁶ The errors of observation associated with each of the various technical procedures used in the determination of metabolism will result in a total observational error that will be included in the estimate of this variability, and therefore also in the intraindividual and total intraindividual variability. The size of this technical error will depend on the methods used and the skill of the technician. It cannot be estimated from numerous observations of simultaneous determinations, as is possible, for instance, in the case of the erythrocyte count, for numerous simultaneous observations of metabolism are impossible. However, an estimate can be made of the variability of each constituent measure used for the determination of the metabolism, and the error resulting from their combination can then be estimated. For the gasometer method in open circuit used for our own observations, this technical error measured as standard deviation amounts in an average case to about 0.2 calorie per square meter per hour. A technical error of these dimensions will have only a negligible influence on the measured biologic variability. On the principle of the addition of independent variabilities, the total variability will be the square-root of the sum of the squares of the constituent variabilities. Thus we would estimate that, if the technical error were entirely eliminated, the measured intradaily variability would be reduced from 0.96 to 0.94 calorie per square meter per hour, and the total interindividual variability from 2.5 to 2.49.

that are included in the total distribution are generally small, a considerable number of the days dealt with having only three observations.⁷

From the data on 3 men given by Benedict and Carpenter we used all the daily observations on the first 15 days. The standard deviation for the 3 men taken together was 1.28 calories per square meter (3.2 per cent of mean). This is somewhat larger than for our series of males.

From the data on Hitchcock in the study by Carpenter, Hoskins and Hitchcock on the effectiveness of deliberate effort to change the basal metabolism, we extracted the observation made on the occasion when no effort was made. The standard deviation for the 4 experimental days taken together was 0.85 calorie per square meter per hour (2.6 per cent of the mean). This is in close agreement with our result for males.

SUMMARY

The variability of basal metabolism, measured as standard deviation in calories per square meter per hour (DuBois) and as relative variability per cent of mean metabolism, has been studied for: 1, the total interindividual variability for subjects of the same sex and age; 2, the interindividual variability of mean metabolism for different subjects of the same sex and age; 3, the intraindividual variability of metabolism observed on different days, and 4, the intraindividual intradaily variability. The quantitative results obtained from our own data and from those of other authors are summarized in table 1.

We found that the intraindividual variability was greater for females than for males, but that the interindividual variability of *mean* metabolism was greater for males than for females. The total interindividual variability in calories per square meter per hour as obtained from single determinations for numerous different individuals (of the same sex and age) is however greater for females than males. This is because the intraindividual variability, which together with the interindividual variability of means makes up whatever is measured in the total intraindividual variability, is enough greater for females to make the net result greater. These intraindividual and interindividual variabilities can be satisfactorily represented by the gaussian or "normal" curve.

The trend and form of distribution of successive intradaily observations was studied. It was found that there was a trend in successive observations, first a decrease, then an increase. While their distribution around the daily means is not gaussian, as observed in accumulations of our data,

⁷ Even if the distribution of deviations of a variate from its mean is strictly gaussian, the distribution of deviations from the means of samples will not be gaussian if the numbers in each sample are small, and this even though the number of samples be indefinitely large.

TABLE 1
Variability of metabolism: summary
 Calories per square meter (DuBois) per hour

	SEX	AGE	NUMBER			VARIABILITY	
			Indi- viduals	Observations	Days	S. D.	Percent of mean
		<i>years</i>					
Interindividual variability, total, age constant:							
Mayo Foundation.....	M	6-19	307			3.14	6.5
Mayo Foundation.....	M	20-64	332			2.58	6.7
Mayo Foundation.....	F	6-19	281			2.98	6.8
Mayo Foundation.....	F	20-17	547			2.42	6.9
Harris and Benedict*	M	20-62	125			2.45	6.4
Harris and Benedict.....	F	20-74	91			2.72	7.7
Harris and Benedict**.....	M	19-44	31			2.83	7.3
Interindividual variability, mean individual metabolism, age constant:							
Mayo Foundation.....	M	20+				2.21	5.8
Mayo Foundation.....	F	20+				1.81	5.2
Intraindividual variability, total:							
Mayo Foundation.....	M	18-40	23	254		1.33	3.5
Mayo Foundation.....	F	18-53	10	137		1.61	4.7
Harris and Benedict†.....	M	20+	6	151		1.43	3.9
Blunt and Dye‡.....	F	21-44	17	221		1.55	4.5
Griffith et al¶.....	M	28-35	2	506		1.18	3.5
Griffith et al.....	F	19-30	3	275		1.34	4.0
Rubenstein§.....	F	23-39	10	295		1.62	4.9
Benedict and Carpenter††.....	M	23-31	3	45		1.65	4.2
Benedict‡‡.....	M	61	1	47		0.73	2.2
Intraindividual variability, intra- daily:							
Mayo Foundation.....	M	26-40	18	1033	445	0.96	2.7
Mayo Foundation.....	F	21	1	33	6	1.11	3.3
Benedict and Carpenter●.....	M	23-31	3	465	111	1.28	3.2
Carpenter, Hoskins, Hitchcock■.....	M	53	1	17	4	0.85	2.6

* Harris and Benedict (2). (Data pp. 40-47. Subjects 20 years and over. Calculations by present authors.)

** Harris and Benedict. (Data p. 234. Calculations by present authors.)

† Harris and Benedict. (Calculated values p. 261, first 6 individuals used. Individuals combined by present authors.)

‡ Blunt and Dye (7). (Data p. 79-85. Calculations by present authors.)

¶ Griffith et al (9). (Calculated values p. 613 used. Individuals combined by present authors.)

§ Rubenstein (10). (Calculated values p. 639 used. Surface area (DuBois) supplied by Rubenstein in personal communication. Individuals combined by present authors.)

†† Benedict and Carpenter. (Data pp. 102-108. First observation each day, first 15 days for each individual used. Calculations by present authors.)

‡‡ Benedict (4). (Data pp. 524-525 used; omitted 4 observations "emotional disturbance"; calculations by present authors.)

● Benedict and Carpenter (11). (Data pp. 102-108. Observations for all days used. Calculations by present authors.)

■ Carpenter et al. (Data p. 321. Normal observations used. Calculations by present authors.)

they are nevertheless symmetrical and the frequencies beyond specific deviations do not depart seriously from what would be anticipated if the distribution were gaussian.

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SOME FACTORS GOVERNING THE INITIATION OF RESPIRATION IN THE CHICK

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Barcroft and Barron (1936) described a generalized rhythmic movement pattern in early sheep fetuses which bears a close resemblance to respiration. Similar respiratory movements have been observed in the rat (Corey, 1932) and rabbit (Snyder and Rosenfeld, 1936) and it is well known that they occur before birth in other animals (Brown, 1915; Windle and Griffin, 1931; Carmichael, 1933), especially when the umbilical cord is pinched. Ahlfeld (1905) called attention to rhythmic respiratory movements in the human fetus which he recorded graphically from the surface of the mother's abdomen. They were found toward the end of pregnancy but diaphragmatic spasms could be identified much earlier. It is not generally known that true rhythmic respiratory movements may be seen in the first half of the human gestation period. However, Dragstedt (1937), watching a fetus of about 14 or 15 cm. C.H. length (4 months, menstrual history) at therapeutic abortion, noticed it gasp and then execute rhythmic respiratory movements for a long time and Erbkam (1837), a century ago, reported movements resembling respiration in a fetus of about the same size. In the sheep fetus, the respiratory-like rhythm subsides toward the middle of the gestation period and reappears when it can be of use shortly before birth.

All factors governing initiation of respiratory movements have not been determined. Corey (1932) believes that carbon dioxide accumulating in the tissues during anoxemia brings about such movements in the rat. Experiments in goats and sheep under urethane led Barcroft (1935) to suggest oxygen want. Neither anoxemia nor carbon dioxide excess stimulated but a minimal carbon dioxide tension appeared to be necessary for maintenance of intrauterine respiratory activity in the rabbit, according to Snyder and Rosenfeld (1936). Henderson (1937) has called attention to the probability that the development of muscle tonus at the time of birth is essential for initiation of pulmonary breathing. Exposure to air,

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drying and other external stimuli are thought to influence the beginning of breathing (Corey, 1931).

The developing chick lends itself well to the study of fetal respiration because observations can be made in normal environment without using depressing surgical procedure and anesthesia. It has however the drawback that it cannot be seen entirely within the egg. Oxygen and carbon dioxide tensions on the maternal side of the mammalian placenta are largely unknown factors but they can be nicely controlled in the incubating chick.

Normal activity in the incubating chick. Movements seen upon transilluminating relatively undisturbed incubating eggs, those observed through opaque shell membrane after removing the shell over the air space, and the activity encountered in the early stages of incubation when the shell membrane is torn away but before the allantois has attached to it are very nearly normal. It is likely that observations made immediately after tearing away the shell membrane and allantois or after dropping oil upon part of the shell membrane are not influenced appreciably by anoxemia. We employed these methods.

The undisturbed chick executed spontaneous movements from about the fifth day on to hatching. Until approximately the fifteenth day, which corresponds to the time the respiratory allantois reaches its maximum growth, movements were frequent, rest periods few. Nothing resembling the early respiration-like rhythm of the sheep fetuses was observed in this period. Correlated with the maturation of the allantois rest periods became longer. Between the fourteenth or fifteenth day and the day before hatching activity decreased; the chick appeared to sleep for long intervals; awaking with series of flutter-like or squirming movements in which sharp kicks and twitches were frequent, only to lapse again into quiet. This was presumably the incubation age at which the chick's oxygen-exchange mechanism functioned most efficiently. During this time it was possible to awaken the chick to greater activity by cutting the shell away from above the air space and blowing upon the shell membrane; perhaps cooling, exposing to light and other manipulations served as well. Churning movements of the amnion were a prominent feature before the fifteenth day but were rarely observed afterwards.

No respiratory rhythm was seen before the seventeenth or eighteenth day (sometimes not before the nineteenth) but movements of all the muscles involved in respiration appeared very much earlier. Simultaneous contractions of abdominal, thoracic, back, neck and jaw muscles corresponding to later individual respirations but lacking the characteristic rhythmicity occurred before the fourteenth day. It is probable that the very earliest extensions of head and trunk seen on the sixth or seventh day are later incorporated in the pattern of respiration. At the time the first definite rhythm was seen only two or three inspirations occurred in

each series of movements; longer and longer rhythms developed as the twentieth day approached. Sometimes deep gasp-like spasms of beak, neck, trunk and leg movements were interposed with other somatic activity. The chick became so energetic in moments of "wakefulness" that it often succeeded in tearing an opening through its membranes into the air space on the twentieth day. Then air-breathing began and a constant but irregular respiratory rhythm was established. Further violent movements led to pipping the shell, usually toward the end of the twentieth day. On the nineteenth or twentieth day the blood vessels of the allantois beneath the air space of the egg were appreciably smaller in diameter than a day earlier. From this time on toward hatching they decreased in size and were torn away when the chick broke out of its shell. But the allantois supplemented the newly functioning lungs before hatching was accomplished.

The effects of oxygen excess. Oxygen in excess altered the movements normally seen in chicks of the last week of incubation. This was especially evident in those whose allantoic vessels had begun to decrease in size and in which the respiratory rhythm was present. The gross squirming or struggling activity was reduced and respiration was either completely inhibited or, if it had been well established, interrupted and much shallower than normal in an atmosphere rich in oxygen. In the early part of the last week of incubation the only effect was a reduction in the amount of head and trunk movement of the kind identified later with respiration. The smaller flutterings of wings and legs were not appreciably affected. In several experiments the pulse rate was a little slower after oxygen had been administered than it had been in air.

Effects of carbon dioxide excess. We tested the effects of various amounts of carbon dioxide at normal pressure usually in an atmosphere containing more than 30 per cent oxygen in 25 experiments with chicks between 13 and 20 days of incubation (before pulmonary breathing had begun). The eggs were placed under a calibrated glass jar inverted over warm water and the gas measured by displacement of water. Most of the observations were made with shell removed above the air space but with the opaque shell-membrane intact. To make sure we were interpreting movements correctly, the shell-membrane was rendered transparent with mineral oil in six and torn away in two instances.

The addition of 0.8 to 1.6 per cent carbon dioxide by volume caused quickening of all movements of nearly all chicks with an increase in amplitude of the contractions of the axial groups of muscles. A brief rapid series of ineffective pecking movements like those used to pip the shell sometimes occurred. Suppression of the finer fluttering of wings and feet was observed with administration of 1.6 per cent carbon dioxide in some and with a higher percentage of the gas in all instances.

The administration of carbon dioxide brought about rhythmic respira-

tory movements in each chick which lacked them normally and in each whose normal rhythm was abolished by an excess of oxygen. In those whose respiratory movements were so far advanced that oxygen merely depressed them carbon dioxide increased the number of rhythmic "breaths" in each sequence and in some instances deepened them and decreased

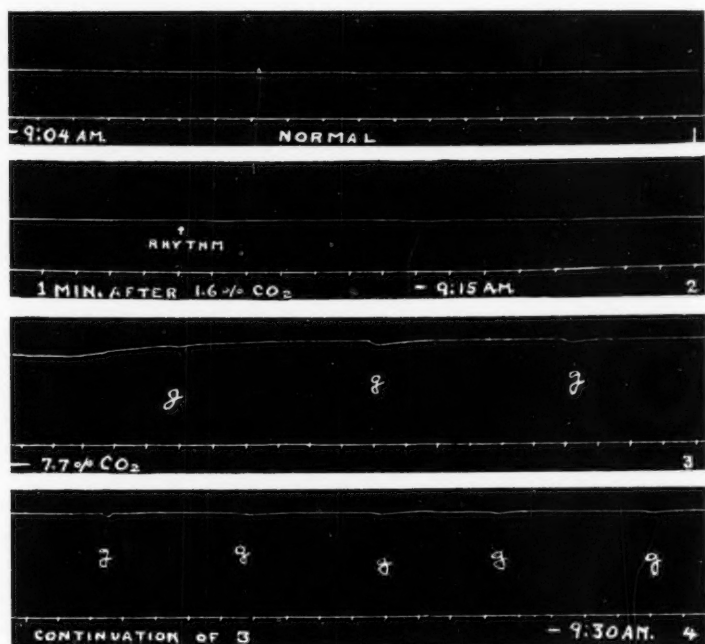


Fig. 1. Chick 96; nearly 18 days; no pulmonary breathing; volume changes in air space of egg transmitted to tambour. 1. During five minutes in air, no rhythmic respiratory movements were seen through glass window sealed into shell over air space. In 34.5 per cent oxygen none was observed in five minutes. 2. After admitting 1.6 per cent carbon dioxide rhythmic respiratory movements which did not cause volume changes began. 3 and 4. After increasing carbon dioxide to 7.7 per cent, gasps occurring in slow rhythm, *g*, were seen through window; these caused reduction of volume as though the lungs were opening. Identical results were obtained in partial anoxemia. Time, 5 second intervals.

their speed. In others the speed seemed to be increased. Approximately 1.6 per cent carbon dioxide brought about respiration in 14 experiments and 0.8 per cent was sufficient in three (undoubtedly it would have proven enough more often had the amount of gas given been limited in the former group). In other experiments definite rhythmic respiratory movements were seen with three per cent and in two of the youngest chicks, five and

seven per cent carbon dioxide. Rhythm was often imperfect in the 13 to 15 day chicks.

Amounts of carbon dioxide greater than 1.6 per cent progressively inhibited more and more movements. At about five per cent gasps sometimes appeared. These were often initiated by a violent struggle. At ten per cent very little movement except the deep respiratory type was observed. This persisted as a rule until the carbon dioxide had reached 25 or 30 per cent. With increasing amounts of carbon dioxide the depth of respiratory movements seemed to increase but the speed of rhythm decreased and ultimately became a series of gasps. The chicks seemed to possess more tonicity of muscles in an atmosphere rich in carbon dioxide. The inspirations were occasionally maintained in a tetanic state for a few seconds.

Carbon dioxide appeared to have little effect upon the pulse rate so long as there was an excess of oxygen. In one experiment the pulse fell slightly below normal but was strong and in another it was definitely fast but shallow in an atmosphere of 30 per cent carbon dioxide. In two others it appeared to be unchanged.

Effects of oxygen want. We administered nitrogen in 21 experiments in chicks between 12 and 19 days of incubation (before pulmonary breathing had begun). In nine of these the shell membrane had been oiled.

Little change in behavior was observed when nitrogen was given gradually until the oxygen had been lowered to about 12 per cent. There was usually a quickening of all movements at this concentration. With any further reduction of oxygen there was a definite increase in neck and trunk muscle contractions and gross squirming or struggling but the finer flutters of wings and feet were less noticeable. In contrast to the almost instantaneous effect of carbon dioxide, changes occurring in a state of partial oxygen-lack usually did not appear for several minutes after a measured amount of nitrogen had been given. A sudden reduction of oxygen to about 15 or 17 per cent brought on quickening of movements.

Administration of nitrogen was less certain than carbon dioxide to produce rhythmic respiratory movements during the first half of the last week of incubation. In most instances a gradual reduction of oxygen to less than ten per cent failed to result in the characteristic respiratory rhythm although nonrhythmic or only imperfectly rhythmic gasps occurred at varying intervals. Chicks between 17 and 19 days of incubation which normally showed incompletely established or nonrhythmic respiratory movements often responded with short rhythms at between 15 and 12 per cent oxygen.

In an atmosphere containing nine or ten per cent oxygen all chicks exhibited depression of those activities not part of the respiratory pattern. But the hypertonic gross trunk movements involved in gasping were

retained and came about at long intervals even when there was less than four per cent oxygen present.

Records of the pulse were obtained in eight experiments. In all but one reduction of oxygen to between 16 and 13 per cent led to an increase in rate and further reduction was followed by a decrease to below normal with irregularities and reduced amplitude of beats. The appearance of a respiratory rhythm seemed to be associated with the drop after the initial rise in rate. The appearance of gasping was associated with further weakening and slowing of the heart.

Effects of obstructing allantoic circulation. At or just before the time the first respiratory rhythms made their appearance normally, destruction of the part of the allantois beneath the air space was followed by an increase in number and amplitude of respirations. Such manipulation at first brought on quickening of all movements, then respiratory rhythms, then depression of finer movements with great increase in tonic struggling and gasping. The gasps sometimes became rhythmic. The results were much the same as those obtained by gradual administration of nitrogen but condensed in point of time.

Tying the allantoic vessels produced even more marked effects. Within two minutes or less marked twitching followed by a gasp and deep rhythmic respiratory movements involving mouth opening (and sometimes eye opening), head and trunk extension and contraction of chest and abdominal muscles occurred; gradually all the muscle contractions dropped out of action save those of the chest. When all rhythm had stopped the chicks were flaccid as though in extreme shock. Untying the vessels was then followed by recovery of the heart action and irritability (providing the heart had not entirely stopped). After a minute or two re-ligation brought on another respiratory rhythm. This could be started time and again in the same individual by tying and untying the ligature upon the allantoic vessels in chicks as young as 14 days of incubation. The youngest chick which gave indication of respiratory movements during anoxemia was ten days old. Chicks whose respiration had been brought to the stage of irregular deep gasps by administering carbon dioxide were unaffected by depriving them of the respiratory allantois. Those depressed by administering large amounts of nitrogen but which had not exhibited marked respiratory rhythm in the low oxygen atmosphere did undergo such a rhythm when the vessels were tied. Ligation of the vessels to the yolk had no effect upon respiration.

The irritability of the chicks to mechanical stimuli seemed to be momentarily heightened and then reduced after tying the allantoic vessels but some recovery followed restoration of the circulation. Muscle tonus developed or increased after ligation and before the respiratory movements began. Sometimes we could see a slow movement like the gradual onset of decerebrate rigidity just before the first gasp.

That the neural mechanism for respiration in the chick fetus involves parts of the nervous system above the spinal cord was proven by severing the upper cervical cord and then tying the allantoic vessels. Rhythmic opening and closing of the mouth without chest respirations resulted.

COMMENT. Our experiments prove that carbon dioxide in low concentration brings about rhythmic respiratory movements in 13 day old chick fetuses although they do not appear normally before 17 or 18 days. Furthermore after the tenth day of incubation rhythmic respiratory movements will start in a state of anoxemia. With intact respiratory allantois, oxygen-want must be acute to induce them and they appear then only after a latent period. There is evidence that some of the respiratory manifestations were associated with slowing or weakening of the heart beat. If the blood flow is slower than normal there is less then normal opportunity for removal of metabolic products. To say that oxygen-want started the respirations is to provide only part of an answer to the fundamental question of what the stimulating agent is. The present experiments demonstrate that solely a low oxygen does not necessarily cause the movements in question to begin.

The pecking movements sometimes started by carbon dioxide administration or appearing in a state of partial anoxemia are thought to be correlated with the initiation of respiration. Normally occurring, they serve to crack the shell so that the chick may start to breath air.

The present experiments may aid our understanding of the relationship between muscle tonus and the initiation of respiration. We believe that administration of a small amount of carbon dioxide or the production of a state of anoxemia either produced or brought about an increase in muscle tonus just before respiratory movements started. This occurred not only at birth but also in the chick a week before hatching. It would seem that carbon dioxide in small amounts stimulates motor centers or releases inhibitory impulses acting upon them whether they be those governing respiration or other movements of skeletal muscle.

The initial result of carbon dioxide administration or of anoxemia was the heightening of irritability and quickening of all somatic movements. With larger quantities of carbon dioxide or with a more advanced state of anoxemia heightened tonus and deep respirations continued although many local movements which are not part of the respiratory pattern were lost. A similar series of events presents itself upon examination of mammalian embryos and small fetuses. For example, reflexes of the forelimbs can be obtained only in the first few moments after opening the uterus (Windle et al., 1935) even though the head and trunk continue to respond to stimulation for some time. This raises a question. Does a depressing amount of carbon dioxide build up in the tissues of the embryo when the abdomen is opened and an incision made through the uterus and chorion although the placenta is still attached to the uterus? Kellogg's (1930)

studies showed a high concentration of carbon dioxide in the blood taken from the heart ventricles upon delivery of dog fetuses by Caesarean section.

SUMMARY

The chick fetus begins to breathe air about 24 hours before it hatches on the twenty-first day. Rhythmic respiratory movements made their appearance normally at about 18 days of incubation. Spontaneous contraction of muscles involved in respiration occur at a much earlier time and in fact the very earliest spontaneous movements of the neck and trunk muscles in five and six-day old chicks continue throughout incubation and seem to be incorporated in the respiratory pattern.

Rhythmic respiratory movements were initiated four to six days prematurely by administration of 1.6 per cent carbon dioxide. A state of oxygen deficiency brought about slowly by administering nitrogen was less certain to stimulate such movements. Anoxemia produced suddenly always did so after a latent period as early as the middle of incubation. The respiratory rhythm which appeared in low oxygen atmospheres seemed to be associated with impairment of heart action.

Both carbon dioxide and anoxemia at first led to an increase of all somatic movements. This period of activity was quickly followed by depression of the finer wing and foot movements leaving only those activities involved in respiration.

The appearance of deep rhythmic respiratory movements seemed to be accompanied by an increase in tonus of the muscles involved in these movements.

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INFLUENCE OF CARBON DIOXIDE AND ANOXEMIA UPON RESPIRATION IN THE CHICK AT HATCHING

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It was demonstrated recently (Windle and Barcroft, 1937) that small amounts of carbon dioxide in an atmosphere rich in oxygen initiated rhythmic respiration-like movements in incubating chick fetuses several days before these movements occurred normally. Similarly results were obtained under partial anoxemia but with somewhat less regularity. The movements in question were short series of rhythmic contractions of thoracic and abdominal muscles alternating with periods of quiet. Greater concentrations of carbon dioxide or more complete anoxemia led to the establishment of stronger respiratory activity resembling the gasping of acute air-hunger. This too occurred rhythmically but the period of the rhythm was slower.

Near the end of the 20th or beginning of the 21st day of incubation the chick perforates its enclosing membranes and begins to breathe the air within the shell. Soon thereafter it chips a hole in the shell admitting atmospheric air. The respiratory allantois continues to function until hatching has been completed. Thus, for a short period the bird employs two respiratory mechanisms. It is with this period that the present experiments are concerned.

METHOD. At about 18 days of incubation a small hole was drilled in the blunt end of each egg and this was surrounded by a wax ring. On subsequent days an egg was quickly removed from the incubator and a capillary glass tube connected with a delicate tambour was applied to this ring. Records of the pulmonary respirations were obtained by means of a long counterbalanced writing lever and a long-paper kymograph which was operated continuously during the course of each experiment. The egg was supported under a calibrated bell-jar over warm water, the level of which could be adjusted to compensate for displacement by gas introduced into the jar. Temperature was controlled by radiant heat. In some experiments a glass window was sealed into the shell over the airspace to provide a view of events taking place within. Figure 1 illustrates the mechanism employed.

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Forty-five experiments were performed upon 20 specimens. Kymographic records suitable for analysis of respiratory rates were obtained in 32 of these. In a few instances the eggs were opened after one or two experiments to determine the condition of the allantois but at least 12 were allowed to hatch. The hatched chicks showed no ill effects of prolonged anoxemia and carbon dioxide excess during the week they were allowed to live.

RESULTS *Normal respiration.* For a short time before the chick takes air into its lungs it normally executes rhythmic respiratory movements

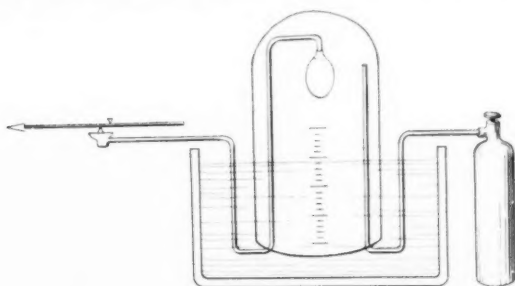


Fig. 1. Diagram to illustrate the method of recording respiration of an unhatched air-breathing chick in controlled atmospheres.

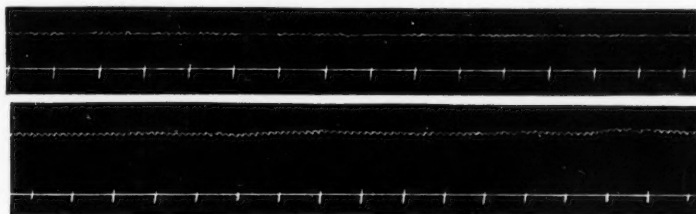


Fig. 2. Upper record illustrates the irregular respiration of an unhatched chick soon after it had begun to breathe air within the shell. Lower record illustrates the normal respiration shortly before hatching. Time: five second intervals.

periodically. We were unable to obtain observations at the moment air breathing began but soon thereafter respiration had become constant although irregular in rate and uneven in amplitude (upper record, fig. 2). Most of the specimens had been breathing air for some time and exhibited regular and even respirations (lower record, fig. 2).

The respiratory rate at the beginning of experiments varied between approximately 55 and 95, with most of the chicks breathing at the rate of 80 to 90 per minute. At the time of hatching respiration was rapid, reaching 100 to 120 per minute in one specimen which had had a rate of

80 to 87 in the shell on the day previously. In the instances in which several experiments were performed with one egg the later records often showed a more rapid rate than the earlier. These observations indicate that pulmonary respiration becomes faster as the time of hatching approaches.

Rebreathing and anoxemia. An increase in respiratory rate sometimes followed sealing the glass tube to an egg. This was most pronounced when much of the shell over the air-space had been covered with wax in attaching the glass window. Perhaps respiration was stimulated by handling the eggs as well as by rebreathing the air within the shell and tambour tube.² In several experiments respiration was unusually rapid at the time recording began but there had been a delay of several minutes between attaching the egg and starting the kymograph and it seemed as though we had

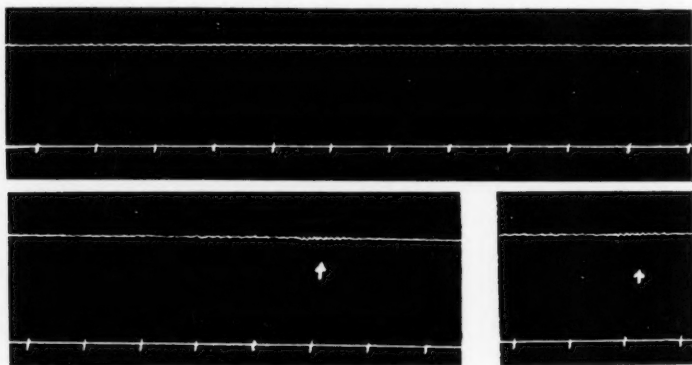


Fig. 3. Upper record illustrates respiration of an unhatched air-breathing chick in an atmosphere containing 47 per cent oxygen. Lower two records illustrate moments of rapid respiration (at arrows) after adding 1.6 per cent carbon dioxide. Compare with figure 7. Time: five second intervals.

recorded only the latter part of a rebreathing effect. The rapid initial respiration usually fell spontaneously to or below what appeared to be a normal level within a few minutes and subsequent reduction of the oxygen in the atmosphere beneath the bell-jar by administration of nitrogen resulted in no significant increase; on the contrary, it led to marked depression below the normal rate in most instances (fig. 10). Similar results were obtained in newly hatched chicks rebreathing within a one-pint jar. The first part of the curve in figure 8 demonstrates respiration stimulated by rebreathing.

² Additional experiments with duck eggs demonstrated that no increase in respiratory rate occurred spontaneously when finely divided sodium hydroxide was placed under the bell-jar and the shell kept clean. It was apparent that there was a free interchange of air between the air space and the bell-jar through the shell.

When the gaseous exchange through shell and shell-membrane was apparently unimpaired to a significant degree the respiratory rate varied

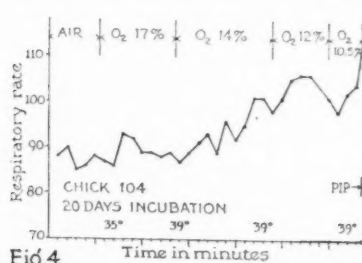


Fig. 4

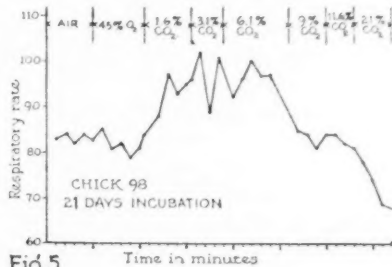


Fig. 5

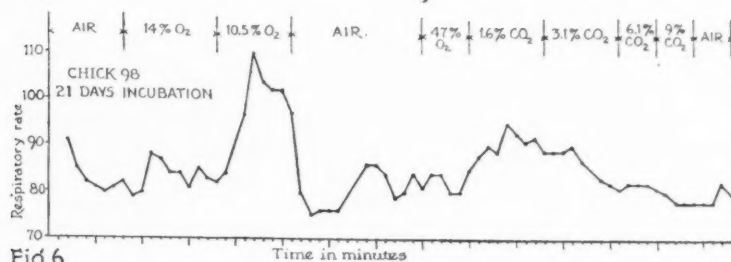


Fig. 6

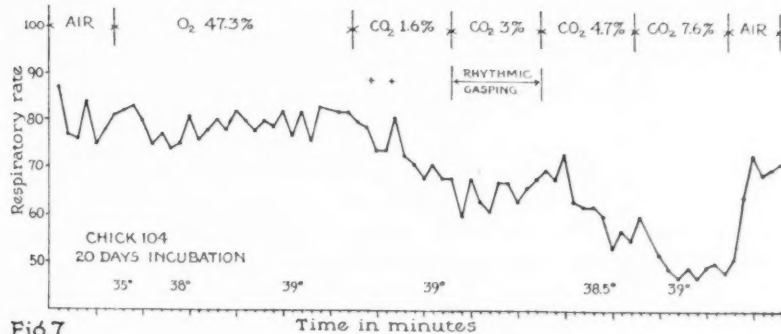


Fig. 7

Figs. 4 to 11. Graphic analysis of respiratory rate per minute interval obtained from kymograph tracings. Each curve illustrates one continuous experiment. The percentage of oxygen or carbon dioxide in the atmosphere breathed by the chicks is indicated above the curves and the temperature of the atmosphere, when recorded, is shown below. In figure 10, brief intervals of very rapid breathing are indicated by the plus signs (see fig. 3); rhythmic gasping was observed through a glass window in the egg. The broken line in figure 11 indicates defects in the kymograph tracing which rendered accurate counting impossible.

but little. One of these specimens showed no increase in rate but five responded with more rapid breathing when the oxygen had been reduced

to between 14 and 8 per cent (figs. 4 and 6). Hatching was sometimes stimulated by lowering the oxygen content of the atmosphere (*PIP*, fig. 4).

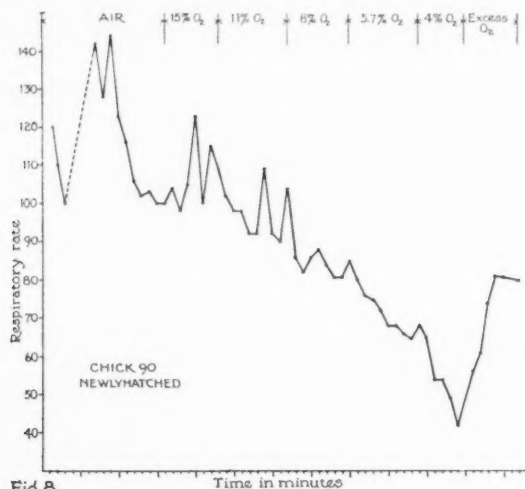


Fig. 8

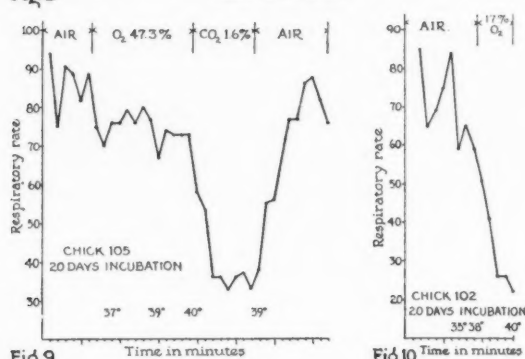


Fig. 9

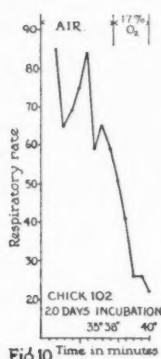


Fig. 10

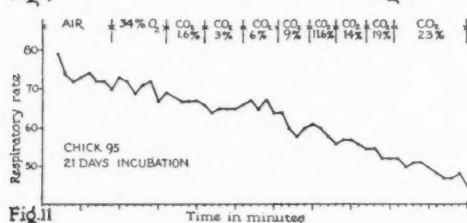


Fig. 11

Effect of oxygen excess. Increasing the oxygen in the atmosphere of the bell-jar to 30-47 per cent had little effect upon the normal respiratory rate

of unhatched air-breathing chicks. When the initial rate was high (re-breathing?) it tended to reduce it somewhat. When low, oxygen administration was sometimes followed by more rapid and even breathing.

Effect of carbon dioxide. In 18 experiments carbon dioxide was added to the oxygen-rich atmosphere beneath the bell-jar from time to time. The effects upon respiratory rate of less than 5 or 6 per cent of this gas were inconsistent. In four experiments low carbon dioxide concentration depressed the respiratory rate sharply (fig. 9). In seven, there were no marked changes when the rate was determined by counting inspirations per minute interval (fig. 11). Definite increase in rate occurred in seven (figs. 5 and 6) but in no case was it so great as that caused by partial anoxemia (fig. 6). When the rate had dropped spontaneously at the beginning of an experiment, oxygen and carbon dioxide tended to restore it to the normal level. Some of the chicks whose respiratory rate appeared to be unaffected or slightly depressed by low concentrations of carbon dioxide (1.6 to 3 per cent) did show brief periods (5 to 15 sec.) of rapid breathing which were followed immediately by slowing; consequently the per minute analysis was not greatly affected. This is indicated in figures 3 and 7.

The concentrations of carbon dioxide in which unhatched air-breathing chicks could maintain vigorous respiration varied considerably. Slower rate and greater amplitude always followed administration of 7 per cent carbon dioxide or more. Some specimens breathed 40 or more times per minute in 30 per cent but in others the rate was reduced to less than 30 per minute in 20 per cent of the gas; one chick exhibited a depression to less than 5 inspirations per minute in 3 per cent carbon dioxide even though the gas mixture contained 45 per cent oxygen.

COMMENT. Interpretation of our results depends upon understanding the conditions which normally obtain in the period preceding hatching. It would seem that so long as the allantois can serve adequately as a respiratory mechanism the chick makes little or no attempt to execute respiratory movements. It was proved (Windle and Barcroft, 1937) that anoxemia and carbon dioxide caused such movements to begin. We suggest that whenever the chick's requirements normally surpass the capacity of the allantois to furnish oxygen and dispose of carbon dioxide movements begin, at first irregularly. Due to the accompanying somatic excitement the shell membranes and later the shell itself are ruptured. The very rapid respiratory rate which the chicks exhibited upon the day preceding hatching may be interpreted as the result of a state of partial anoxemia with attendant accumulation of carbon dioxide or of metabolic products. Such a state is correlated with the drying up of the allantois at the time of which greater dependence is placed upon the lungs.

At hatching the lungs are rather suddenly forced to take over the entire

respiratory function. It seems probable that the speed of respiration manifested at this time depends upon the efficiency of the newly functioning lungs. The results obtained by submitting unhatched air-breathing chicks to low-oxygen atmospheres or to atmospheres containing an excess of carbon dioxide may depend upon the reserve capabilities of the allantois and the lungs. If there is no reserve there will be no further increase in respiratory rate but on the contrary there may be a marked decline comparable to that seen in acute oxygen-want in the adult. In those chicks which are meeting the crisis of hatching with adequate reserve a slight amount of carbon dioxide or a reduced supply of oxygen will produce more rapid respiratory efforts.

Although the foregoing explanation seems to account for many of our results adequately other factors may be involved. Because the allantois lacks nerve fibers carbon dioxide must act by way of the blood stream to produce stimulation of the respiratory centers. Is the effect duplicated when carbon dioxide enters the lungs and reaches newly functional nerve endings? It is possible that nerve impulses from the lung receptors modify and partially counteract humoral stimulation of respiratory centers by carbon dioxide. This would explain our experiments in which small amounts of carbon dioxide led to marked respiratory slowing. Furthermore, in no experiment was the increase in respiratory rate so great with carbon dioxide in excess oxygen as it was without carbon dioxide and with deprivation of oxygen. Since carbon dioxide proved to be a more efficient respiratory stimulus than anoxemia before the lungs function (Windle and Barcroft, 1937), it is difficult to explain these differences purely upon a humoral basis. It is clearly suggested that the lungs have contributed some new factor.

CONCLUSIONS

1. Respiratory movements, irregular and periodic before, become regular and continuous soon after the chick begins to breathe air. This occurs about one day before hatching is completed.

2. The respiratory rate of unhatched air-breathing chicks increases as the time of hatching approaches. Drying up of the allantois is an associated phenomenon. It is suggested that rapid respiration at hatching is an expression of anoxemia and carbon dioxide accumulation occurring normally.

3. Oxygen-want and apparently rebreathing within the sealed shell served to increase the respiratory rate in some unhatched air-breathing chicks. However, if the chick's respiration were very rapid at the beginning of an experiment (rebreathing?), reduction of oxygen usually depressed it.

4. A small amount of carbon dioxide in an excess of oxygen had a stimulating effect upon the respiratory rate in some but had no effect or else depressed the rate in other chicks. Carbon dioxide in concentrations greater than approximately 6 per cent slowed respirations and increased their amplitude.

5. It is suggested that two factors influencing the respiratory phenomena observed are: *a*, the anoxemia and carbon dioxide accumulation accompanying physiologic decline of the respiratory allantois at hatching, and *b*, the stimulation of newly functioning inhibitory lung receptors by carbon dioxide.

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DEVELOPMENT OF RESPIRATION IN THE DUCK

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Rhythmic respiratory movements of fetal chicks were readily induced by administering carbon dioxide or by obstructing the circulation through the allantois but the use of nitrogen to produce oxygen deficiency appeared to have little effect until the heart action had become impaired (Windle and Barcroft, 1937). A state of partial anoxemia with carbon dioxide accumulation which occurs normally as the respiratory allantois deteriorates on the day preceding hatching apparently initiated and accelerated pulmonary breathing. In air-breathing unhatched chicks administration of either carbon dioxide or nitrogen accelerated the respiratory rate of some and depressed it in others depending, it seemed, upon the degree of anoxemia normally obtaining (Windle, Scharpenberg and Steele, 1937). The possibility that stimulation of newly functioning inhibitory lung receptors influenced results in atmospheres containing carbon dioxide was considered.

The adult duck possesses a reflex mechanism which functions to suspend respiration and reduce oxygen consumption while diving (Huxley, 1913). It was reported that carbon dioxide brought about slowing of respiration or complete apnea although oxygen want served as a direct excitatory stimulus causing acceleration of the rate (Orr and Watson, 1913).

METHODS. The present series of experiments in duck fetuses was projected to determine whether respiratory responses conform to those obtained in the fetal chick or the adult duck and if possible to learn the meaning of significant differences. Experimental methods were similar to those used for the chick (Windle, Scharpenberg and Steel, 1937). In addition to direct observations in intact transilluminated eggs and those with shell removed above the air space, experiments were performed in the following manner. A capillary glass tube was sealed to a hole drilled in the blunt end of each incubated egg. It was led to a delicate tambour with long counter-balanced writing lever. The egg was supported over water under a calibrated bell-jar into which gases were admitted while the water levels were adjusted to maintain equal pressures. Temperature was controlled. Respiratory movements of the unhatched ducks breathing through porous shell and membranes were recorded on smoked paper. After the specimens had chipped a hole in the shell it was sometimes, although not always,

¹ Aided by a grant from The American Academy of Arts and Sciences.

necessary to seal this with wax. Thus, experiments in duck breathing the atmosphere within the shell were controlled by others in which the fetuses breathed the contents of the bell-jar directly.

Respiratory movements before the lungs function. The incubation period (at 102°F.) of the Indian Runner and White Pekin eggs used was about 28 days. Hatching began at 25 or 26 days. Rhythmic respiratory movements could not be detected by transillumination until three days before the end of incubation but irregular and arrhythmic movements which undoubtedly involved respiratory muscles increased in number about a day earlier. When the shell above the air space had been removed arrhythmic respiratory movements were observed as early as the twenty-second day.

Upon setting up a state of partial anoxemia by tearing away portions of the shell membrane and allantois, rhythmic movements of the beak and body followed as early as 21 days. Pinching the allantoic vessels resulted in distinct respiration-like rhythms at 11 days of incubation. The sequence of activities was similar to that reported in the chick (Windle and Barcroft, 1937).

Eggs with shell removed above the air space were placed in air containing 47 per cent oxygen under a bell jar over water and measured amounts of carbon dioxide were added. Rhythmic movements of respiratory muscles were induced in 13 to 23 day old duck fetuses by this means. As little as 1.6 per cent carbon dioxide quickened somatic activity but rarely excited a rhythm. Brief rhythms of strong and regular respiratory movements always appeared after adding 3 to 6 per cent carbon dioxide. In 10 to 20 per cent of the gas the fetuses gasped at regular intervals (about 10 to 20 sec.) and all somatic behavior was depressed except at moments of gasping. The only significant difference between duck and chick fetuses seems to be one of threshold to carbon dioxide stimulation; the chick responded at lower concentrations than the duck.

Respiratory activity of unhatched air breathing ducks. Respiration is carried on both by the allantois and lungs for about 3 days before hatching is completed. Because this period is three times as long in the duck as it is in the chick it was possible to study a specimen repeatedly during hatching and afterward. Long-paper kymograph records of 35 experiments with the effects of carbon dioxide and oxygen want were analyzed graphically. Some of these are presented in figures 2 to 5.

Initiation of air breathing. Pulmonary respiration began about the twenty-sixth day of incubation at rates of 20 to 50 per minute. It was irregular at first and each imperfect rhythm lasted only a few seconds. Quiet intervals of several seconds or minutes followed each respiratory attempt. Breathing soon became continuous and usually had assumed a fairly even rate by the end of the twenty-sixth day. The normal respiratory rate accelerated spontaneously from approximately 60 to 110 or more

breaths per minute between 26 and 28 days. Before some of the ducks had completely hatched the rate diminished to 50 or 75 per minute. Most individuals breathed 30 to 45 times a minute when undisturbed during the

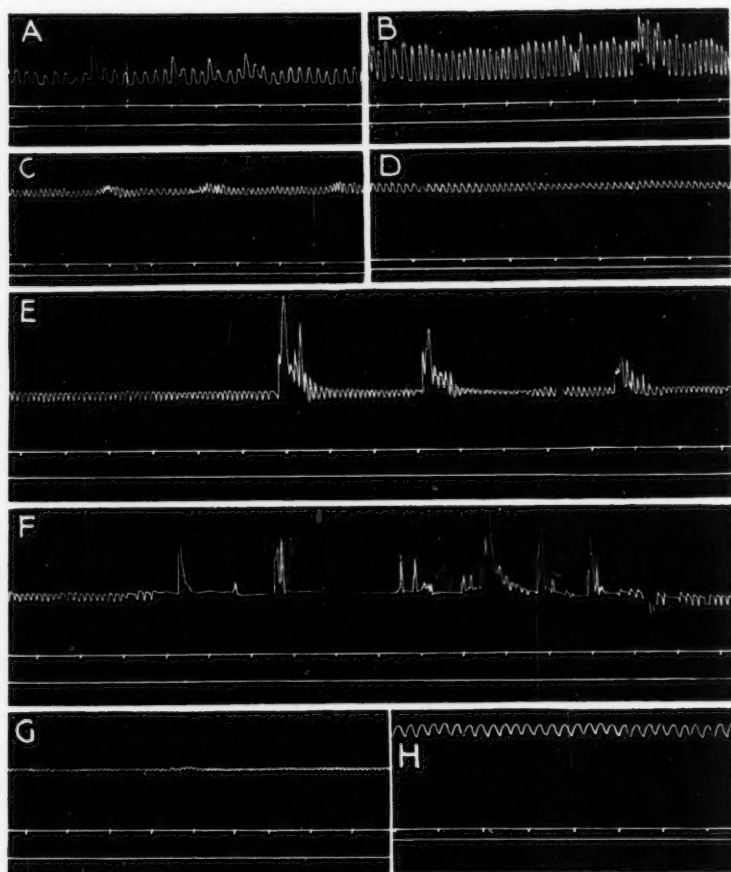
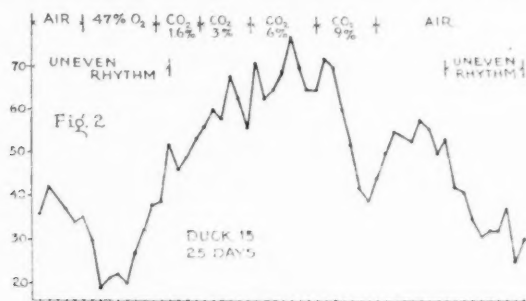


Fig. 1. Records of respiration in unhatched air breathing duck 33 and in a newly hatched duck. Inspiration is the up-stroke. Time: 5-second intervals. A: normal respiration of 26 day duck fetus 33. B: respiration of the same specimen after reducing the oxygen to 14 per cent. C: rebound acceleration in air after administering 10 per cent carbon dioxide in excess oxygen to the same specimen. D: normal respiration of the same fetus a day later (27 days). E and F are continuous and illustrate respiration of the 27 day specimen in 4.7 per cent carbon dioxide with 47 per cent oxygen; E shows periods of excitement and F illustrates the inhibition and slowing of respiration by the carbon dioxide entering the lungs. G: rebound acceleration in air after administering 7.5 per cent carbon dioxide in excess oxygen to the same 27 day old specimen. H: normal respiration of a one day old duck.

week following hatching (up to 80 per minute when excited). The respiratory rate of adult ducks varies between 6 and 30 per minute (Orr and Watson, 1913). Comparison of A, D and H in figure 1 illustrates the spontaneous rise and decline of the respiratory rate at hatching.

Effects of carbon dioxide. Administration of carbon dioxide in an excess of oxygen to a 25-day old duck fetus whose normal respiration was interrupted and imperfectly rhythmic resulted in continuous and rhythmic breathing almost immediately (fig. 2). The respiratory rate was accelerated by carbon dioxide in concentrations of 1.6 to 6 per cent but depressed by 9 per cent. Upon returning to air, breathing again became uneven after a few minutes.

The respiratory rate of 25 to 26 day old specimens which had established continuous breathing at a rate of 55 to 80 per minute accelerated under the

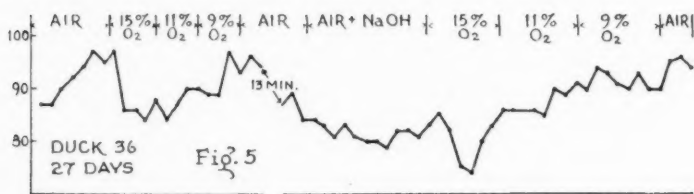
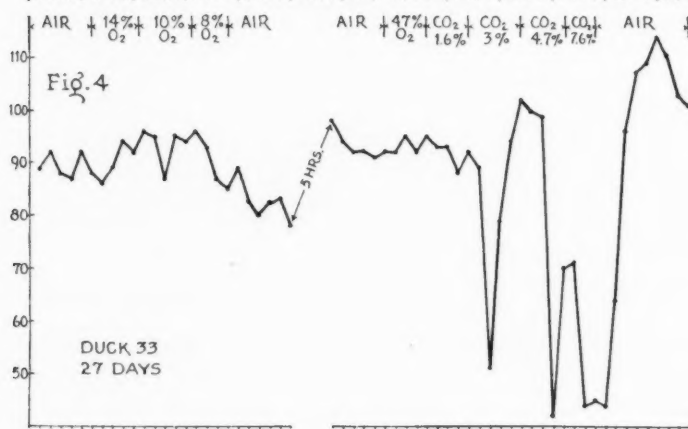
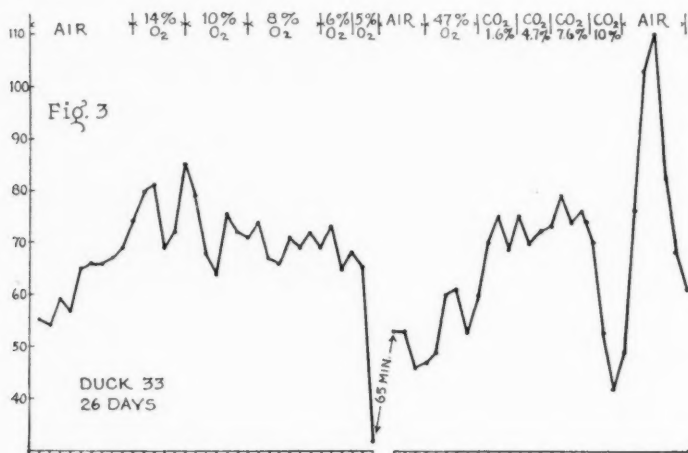


Figs. 2-5. Graphic analyses of records of respiration in unhatched air breathing ducks. The nature of the atmosphere breathed is indicated above, respirations per minute on the ordinate and time in minutes on the abscissa. Figure 2 illustrates a fetus at the beginning of lung respiration which was intermittent for half an hour before the first point on the curve. Figures 3 and 4 are from one specimen 23 hours apart. Figure 5 demonstrates re-breathing and oxygen deficiency effects.

influence of atmospheres containing 47 per cent oxygen and less than 8 per cent carbon dioxide (fig. 3). Subsequently, 10 per cent or more carbon dioxide invariably produced slowing and sometimes momentary cessation of breathing. Upon returning to air a marked rebound acceleration occurred; frequently the rate rose to a higher level than during carbon dioxide administration but returned to normal in a few minutes (figs. 1C and 3).

After normal respiration had reached a high rate at 27 to 28 days of incubation, 3 to 6 per cent carbon dioxide produced no sustained acceleration. Sometimes moments of more rapid breathing alternated with periods of slowed or inhibited respiration (figs. 1E, F and 4). Higher concentrations of carbon dioxide resulted in marked respiratory depression as before. Frequently 5 per cent or less brought about inhibition and a sharp decline in the respiratory rate. The rebound after returning to air was usually marked (figs. 1G and 4); in two instances it attained a peak

exceeding 120 per minute although the administration of carbon dioxide had induced no pronounced rise.



In the period just before hatching was completed, when the respiratory rate had become slower normally, administration of small quantities of

carbon dioxide tended to elevate the general level of the respiratory rate. But moments of slowed or inhibited breathing occurred in 5 per cent and 8 to 10 per cent induced very pronounced depression. Acceleration such as observed on the twenty-sixth day, at the beginning of pulmonary function, was definitely masked by a strong inhibitory action.

Rebreathing effect. A spontaneous and transient acceleration of the respiratory rate frequently took place at the time an incubated egg was being connected to the recording mechanism. This was probably due to mechanical stimulation for if allowed to remain undisturbed in the open air the fetus established a lower rate at which breathing remained constant. However if the bell jar were placed over the egg and water levels adjusted so that the jar contained two liters of air, the respiratory rate often began to rise within two or three minutes and remained high until the volume of air was increased by adding more air, oxygen or nitrogen (figs. 2 and 5). That this acceleration resulted from accumulation of respiratory carbon dioxide was indicated by the fact that finely divided sodium hydroxide placed beneath the bell jar prevented or abolished the effect (fig. 5). A free and rapid interchange of gases apparently took place through the shell membranes and shell of the air space providing the shell had been kept clean and free from excess wax used to attach the tambour tube.

Effects of oxygen want. The effects produced by reducing the oxygen concentration by adding nitrogen to the air beneath the bell jar were observed in 13 experiments. Three 26 to 27 day ducks showed small sustained acceleration of respiratory rate in 14 to 9 per cent oxygen (figs. 1B and 3); others were unaffected or there occurred moments of transient rapid breathing followed by slowing. When the initial respiration was rapid at 27 to 28 days similar reduction of oxygen either had no effect or else depressed the rate. In some instances 9 per cent oxygen brought about collapse of the respiratory mechanism and 5 per cent nearly always did so. With finely divided sodium hydroxide placed beneath the bell jar, oxygen reduction sometimes accelerated respiration when the initial rate was 85 per minute or less (fig. 5), but failed to do so when it was 100 to 110 per minute. The rebound acceleration seen after depression of the rate with carbon dioxide was not encountered in experiments with oxygen want.

Respiration in newly hatched ducks. Two ducklings were removed from the shell on the day hatching was expected. In one instance the allantois had dried and did not bleed. This specimen showed momentary excitement but breathing quickly resumed approximately the rate established before removal. In the other, the allantois was still moist and a significant volume of blood was flowing through it. Its severance was followed by prolonged excitement and the respiration rate was maintained at a high level for nearly half an hour. In both specimens, as in all the 12 other 1 to 10 day old ducklings so tested, administration of 5 to 10 per cent carbon

dioxide in oxygen by a thistle tube placed over the head was followed by inhibition of breathing or marked slowing of the respiratory rate. Acceleration was never encountered. However upon returning to air a period of very rapid respiration occurred (the rebound effect). Administration of nitrogen containing 5 and 7.5 per cent oxygen to ducklings resulted in acceleration of the rate considerably above that of normal breathing.

COMMENT. The experiments with duck fetuses confirm observations in the chick and allow us to conclude that carbon dioxide accumulating during the state of partial anoxemia, which ensues while the fetus is outgrowing its respiratory allantois toward the end of incubation, is the principal factor governing the initiation of respiratory movements. Such movements were less readily obtained in chick fetuses submitted to atmospheres deficient in oxygen than in those subjected to a small excess of carbon dioxide (Windle and Barcroft, 1937). Only with deterioration of the heart action did distinct rhythms of respiratory movements ordinarily manifest themselves under the influence of oxygen lack. On the other hand the acceleration of respiratory rate of the air breathing unhatched ducks placed in a lowered concentration of oxygen with sodium hydroxide to absorb expired carbon dioxide suggests that oxygen want can facilitate initiation of breathing.

The natural progressive acceleration of the respiratory rate during the three days terminating incubation is clearly correlated with the progressive deterioration of the allantois. The lungs supplement allantoic respiration when first called upon and take over more and more of that function as hatching advances. The decline of the respiratory rate observed in some instances upon the last day of incubation indicates that the duckling had passed a crisis in the process of transferring from the allantoic to lung respiration and was able to give off carbon dioxide and take in oxygen more efficiently.

We can not support the theory of Paton (1913) that carbon dioxide produced apnea by acting upon the respiratory center by way of the blood stream. When this gas enters the blood stream through the allantois, an organ containing no nerves, it invariably provokes rapid respiratory rhythms. After the lungs begin to function the excitatory effect persists until the fetus reaches the stage at which the natural rate of breathing (in the anoxemia of hatching) nearly equals that under the influence of carbon dioxide. It is only when drawn in by the lungs that carbon dioxide produces slowed breathing or apnea. While allantois and lungs are both functioning the accelerating effect of carbon dioxide acting through the former is tempered or abolished by an inhibitory effect through the latter with the result that the gas seems to have produced little or no change in the rate of breathing. However upon returning such a fetus to air a marked rebound acceleration ensues because the lungs no longer receive

carbon dioxide and the accumulation of this substance in the tissues has a chance to act unopposed upon the respiratory centers.

CONCLUSIONS

1. Carbon dioxide excites acceleration of respiratory rate when it acts by way of vascular channels but produces marked inhibition and slowing of respiration when taken into the lungs of unhatched air-breathing ducks.

2. Deficiency of oxygen in the inspired air results in acceleration of the respiratory rate.

3. Pulmonary respiration, which normally starts about 3 days before the end of incubation, appears to be initiated by the excitatory effect of metabolic carbon dioxide accumulating during the state of partial anoxemia which accompanies the physiologic deterioration of the respiratory allantois. The initiation of respiration may be facilitated by the excitatory action of oxygen deficiency.

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SOME DETERMINATIONS OF THRESHOLDS TO STIMULATION WITH THE FARADIC AND DIRECT CURRENT IN THE BRAIN STEM¹

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In excitation work from this laboratory (Kabat, Magoun and Ranson, 1935; Magoun, Hare and Ranson, 1935; Magoun, Ranson and Fisher, 1933; Ranson, Kabat and Magoun, 1935; Ranson and Magoun, 1933a and Ranson and Magoun, 1933b), the brain stem has been stimulated routinely with submaximal induction shocks. No attempt was made to ascertain thresholds for the responses at that time, but in order to rule out any possibility that our results were due to spread of current, determinations of thresholds of several parts of the brain stem have been undertaken. Concurrently comparisons were made using faradic current, rectangular direct current and the "dampened" direct current advocated by Hess (1932). Qualitative observations were made on the effects of changes in rate of stimulus on threshold and nature of response. Differences in the action of nembutal and urethane as anesthetics were noted during this study of some 39 cats.

METHODS. A mechanical interrupter was used for controlling the frequency and duration of stimulation and for shorting out make faradic shocks. The duration of the direct current was usually one-fourth to one-fifth of the cycle. The direct current intensity was controlled by a two meter slide wire of 27 ohms resistance on a scale marked in millimeters. The current was obtained from a 12.6 volt storage battery whose exact voltage was periodically checked with a volt-meter accurate to 2 per cent.

For "dampening" the direct current, circuit constants were chosen to approach those of Hess. Two coils from a Model T spark coil secondary with direct current resistances of 1,700 ohms each and a 4 mfd. condenser were used. When arranged as described by the above author, the form of the current was such as to rise to 95 per cent of maximum in 16 msec. This value was obtained by measurement of photographic records of a cathode ray oscillograph.

The primary of the inductorium was used with the iron core in place, and the secondary coil was shunted by either a 2,000 or 10,000 ohm resistance to increase the duration of the shock. Curves were plotted with

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peak height of induction shocks in volts against the distance of separation of the primary and secondary coils.

The electrodes used in the brain were the same bipolar electrodes used by us routinely in other stimulation with the Horsley-Clarke stereotaxic instrument with tip separations 1 or 2 mm. along the long axis of the wires. In all but the earlier experiments the nichrome electrode tips were thinly plated with copper, heavily silver plated and chlorided electrically. Platinum electrodes 5 mm. apart in a bakelite shield were used for stimulation of peripheral nerve.

The sites of stimulation in the brains of representative animals were checked histologically to see the effects of the various current types on the tissues. The brains of these animals were fixed in 10 per cent formalin, embedded in celloidin and 50 micra sections cut serially. Every fourth section was stained by the Weil technique for nerve fibers and one adjacent to it with cresyl violet for nerve cells.

Observation of pupillary reactions was aided by a small telescope magnifying 3.5 times.

The experimental procedure adopted was to determine the threshold to induction shocks at 12 per second; then thresholds to induction shocks at 20, 16, 12, 8, 6 and 4 per second; and finally at 12 per second. Next rectangular direct current stimuli were tested at 20, 16, 12, 8, 6 and 4 per second and the threshold to induction shocks at 12 per second again determined. The same procedure was followed with modified direct current stimuli. Induction shocks at 12 per second were a control to detect threshold differences if there should be any due to polarization of electrodes or electrolysis of tissues.

The time of application of a stimulus before a determination was considered negative was sometimes as long as 25 seconds to allow the response to develop. Knoeffel and Davis (1933) stress the use of adequate periods of stimulation.

Results of stimulation of the hypothalamus. Pupillodilatation. This was bilateral although the readings were made only on the right pupil. The response was obtained easily under all of the anesthetics used. The parts of the hypothalamus from which the response can be obtained have been described by Ranson and Magoun (1933b) and Ranson, Kabat and Magoun (1935) and need not be repeated here.

The thresholds to faradic stimulation using a 2,000 ohm secondary shunt are given in table 1; taking 12 per second as standard, the table also shows the relative thresholds at various frequencies.

As values for comparison with the voltages given in this paper, the peak voltage of the shocks produced during one calibration of a Harvard inductorium with 1.5 volts in the primary circuit, 10,000 ohms resistance in the secondary circuit, and a coil separation of 11 cm., is 16.5 volts and of

12 cm. is 13.5 volts. Increasing the resistance in the secondary circuit increases the peak value of the shock but decreases its duration. The voltages given are with an iron core in the primary, removal of which will decrease the voltage and decrease the duration of the shock.

The measure of variability used is the approximation to the standard deviation for a small sample, s , because the series of 9 cats used for this particular determination was small (Fisher, 1934; and Scott, 1927). It will be noticed that the values of s are so large that no significance would ordinarily be attributed to the apparent increase of threshold as the frequency is decreased. The effect that frequency has on threshold, however, is masked by the variation of threshold to the same frequency from

TABLE 1

FREQUENCY	THRESHOLD IN VOLTS	s	RELATIVE THRESHOLD	s
20/sec.	3.35	0.81	0.86	0.13
16	3.58	0.68	0.91	0.04
12	3.86	0.77	1.00	
8	4.56	1.26	1.16	0.22
6	4.98	0.94	1.28	0.19
4	7.85	2.42	2.02	0.65

TABLE 2

FREQUENCY	THRESHOLD IN VOLTS TO RECTANGULAR DIRECT CURRENT PULSES		THRESHOLD IN VOLTS TO MODIFIED DIRECT CURRENT PULSES	
		s		s
20	0.93	0.38	1.55	0.61
16	1.01	0.41	1.61	0.70
12	1.11	0.45	1.70	0.67
8	1.28	0.42	1.87	0.81
6	1.41	0.42	2.06	0.82
4	1.54	0.52	2.35	0.97

animal to animal, but the steady increase of threshold with decreasing rate shown in the table can scarcely be due to chance. Since a threshold to each frequency was determined at each point stimulated, corresponding values at the various frequencies can be compared by the method described by R. A. Fisher for the significance between the means of small samples in a controlled comparison (p. 118). It can thus be shown statistically that the differences in threshold between frequencies of 20 and 12, 16 and 12, 12 and 8, 8 and 4, and 6 and 4 are valid differences.

Stimulation of the hypothalamus with direct current stimuli with the animal under nembutal and using pupillodilatation as an indicator resulted in the thresholds shown in table 2.

As will be shown later, anesthetics had very little effect on thresholds for pupillodilatation, whereas they affected retraction of the nictitating membrane and piloerection very greatly. The assumption that perhaps the same mechanisms were not involved was tested by trying the effect of severing the cervical sympathetic trunk on threshold of pupillodilatation.

Cutting the cervical sympathetic trunk results in a more marked constriction of the pupil on the same side than existed before, so that the threshold obtained with it in this condition may not justifiably be compared with that of the same pupil before severance of the trunk or with the pupil of the opposite side. However, the frequency-threshold values as well as the absolute thresholds obtained when only inhibition of pupilloconstriction was responsible for the pupillodilatation were essentially the same as when the cervical sympathetic was intact—this holds when nembutal is the anesthetic but when urethane was used the thresholds on the sympathetomized side were perhaps a little higher than those on the normal side, but the differences were not great enough to be conclusive.

TABLE 3

FREQUENCY	INDUCTION SHOCKS THRESHOLDS IN VOLTS	RECTANGULAR DIRECT PULSES	MODIFIED DIRECT CURRENT PULSES
20	2.2	0.55	1.01
16	2.3	0.65	1.16
12	3.0	0.83	1.39
8	3.6	1.24	1.83
6	5.0	1.39	2.59
4	8.9	1.97	2.69

Nictitating membrane. A response of this structure was very difficult to elicit with the animal under nembutal, was present but with a rather high threshold in the one animal under "Dial," and was regularly present under urethane. Since there were but 4 cats anesthetized with urethane in this series no attempt was made to use statistical methods in interpreting the data.

Arithmetical averages of the results under urethane are shown in table 3.

Piloerection. Erection of hair over the back and tail was not routinely looked for but in 2 experiments under urethane it was noticed, so a series of induction shock thresholds was determined in each case. The averages of the two series at the 6 frequencies normally used were: 4.7, 5.0, 6.7, 9.0, 15.7 and 37 volts.

Stimulation of the posterior commissure. Stimulation of the posterior commissure resulted in bilateral pupilloconstriction as has previously been reported (Ranson and Magoun, 1933a), with no concomitant movements of the eyeballs. The response was very sensitive to degree of anesthesia,

so that numerical averages with the mean deviations of the thresholds have no practical significance. The only method of presentation of value is to give the series of thresholds at the various frequencies and state the dose of anesthetic. Even this is inadequate because the depth of anesthesia of different animals varies with the same dose of anesthetic. In one animal which received a dose of 1.5 gram urethane per kilogram the posterior commissure had the following thresholds to induction shocks at the six usual frequencies: 1.7, 1.7, 1.9, 2.2, 2.3, 2.2 volts. The effect of frequency on threshold was slight. If relative values are considered rather than absolute ones, results obtained on 9 other animals were similar.

Stimulation of the oculomotor nerve. The oculomotor nerve was stimulated in the brain stem as it passed downward from the third nucleus toward the base of the brain. The two responses elicited were twitching of the eyeball and upper lid at the rate of stimulation and constriction of the pupil of the same side. The constriction of the pupil reflected the rate of stimulus at rates of 1 or 2 per second or with single shocks, but at rates

TABLE 4

FREQUENCY	FARADIC IN VOLTS	s	RECTANGULAR DIRECT	s	MODIFIED DIRECT
20	0.9	0.49	0.4	0.2	0.9
16	0.9	0.52	0.4	0.2	0.9
12	0.9	0.52	0.5	0.2	1.1
8	1.1	0.65	0.6	0.2	1.1
6	1.5	0.75	0.75	0.3	1.5
4	1.9	0.9	0.9	0.4	2.1

above 4 per second the constriction was steady throughout the period of stimulation.

The threshold for twitching of the eyeball depended on the position of the electrode tips but was apparently independent of depth of anesthesia or rate of stimulation within the ranges employed. The induction shock threshold was 1.5 (s 0.4) volts (12 cats) using a 10,000 ohm shunt across the secondary; the rectangular direct threshold was 0.8 (s 0.4); and the modified direct current threshold was 3.0 (s 0.8).

Just 4 complete series of induction shock thresholds were run using pupilloconstriction as an indicator. The averages of the 4 series at the 6 usual frequencies are: 2.2, 2.1, 2.3, 2.2, 2.3 and 2.4 volts. The averages of 2 series with rectangular direct current stimuli are 0.99, 0.99, 1.04, 1.12, 1.24 and 1.39 volts. Using direct current, there appears to be some effect of frequency on threshold, but using induction shocks the effect was masked by variation at each frequency. Before it was realized that stimuli at the several frequencies should be applied at the same point in the

brain in order to have a controlled comparison, the oculomotor nerve was stimulated with rectangular direct current in 5 cats at each of 3 frequencies. The thresholds were 1.3 (s 0.7) at a frequency of 4 per second; 0.9 (s 0.4) at 8 per second; and 0.8 (s 0.3) at 16 per second. The significance of the difference between the means is lost, however, because of the small size of the number of observations and the large value of the standard deviations.

Stimulation of the cervical sympathetic trunk. With platinum electrodes separated by 5 mm. along the axis of the nerve and covered by a small bakelite shield the thresholds shown in table 4 were obtained, using retraction of the nictitating membrane as an indicator.

Thresholds for pupillodilatation were slightly but not statistically higher.

Stimulation of the sciatic nerve. Threshold studies were made on the

TABLE 5

	INDUCTION SHOCKS IN VOLTS	RECTANGULAR DIRECT IN VOLTS	MODIFIED DIRECT IN VOLTS
Minimum thresholds.....	0.04	0.026	0.24
Average thresholds.....	0.15	0.09	0.75
Maximum thresholds.....	0.3	0.25	1.50

TABLE 6

	INDUCTION SHOCKS IN VOLTS	RECTANGULAR DIRECT IN VOLTS	MODIFIED DIRECT IN VOLTS
Minimum threshold.....	5	1.5	3.6
Average threshold.....	20	3.0	5.8
Maximum threshold.....	64	7.4	9.0

sciatic for purposes of comparison with the other thresholds that we obtained. Within the range of frequencies studied there was no detectable frequency effect on threshold. Using platinum electrodes 5 mm. apart along the axis of the nerve, the thresholds shown in table 5 were found with plantar flexion of the hind foot as the indicator.

Stimulation of the motor cortex. Thirteen experiments were done on the motor cortex under nembutal anesthesia. The shunt used on the secondary of the induction coil was 10,000 ohms. The effect of frequency was not on the absolute threshold but rather on the nature of the response, whereas the duration of stimulus markedly affected the threshold values.

Stimulation of the lateral part of the left anterior sigmoid gyrus elicited twitching of the right fore limb. Thresholds for this part of the cortex are given in table 6.

Twitching of the forelimb occurred at the rate of stimulus up to 8 per

second with relaxation of the limb between contractions. Up to 16 stimuli per second the rate of contraction still reflected the rate of stimulation but the limb did not completely relax between shocks, while above 16 per second the flexion was tonic or else walking or stepping movements occurred at 1 to 4 per second. With repeated stimuli the threshold sometimes became lower; continued application of initially subthreshold stimuli would occasionally cause a reaction in 5 to 12 seconds; or continued application of initially threshold stimuli resulted frequently in increased excursion of the contractions at low frequencies or increased the rate of stepping at higher frequencies. Ward and Clark have reported responses of various cortical areas in the cat to alternating current stimuli (1935).

Stimulation of the internal capsule. The middle part of the left internal capsule about 5 mm. from the midline was used because in most cases flexion of the right fore-limb could be elicited from this point. Fifteen cats under nembutal were used in this series. Nembutal definitely depressed the reactions and raised the threshold to stimulation of the internal capsule, but under urethane a response could not be elicited at all.

TABLE 7

	INDUCTION SHOCK IN VOLTS	RECTANGULAR DIRECT IN VOLTS	MODIFIED DIRECT IN VOLTS
Minimum threshold.....	3.0	1.2	1.5
Average threshold.....	15.2	2.6	4.0
Maximum threshold.....	66.0	6.0	9.8

"Dial" was tried also but results were unsatisfactory. Thresholds obtaining were as indicated in table 7.

Stimulation at rates up to 8 per second elicited flexion of the right fore-limb at the same rate with relaxation of the limb between shocks. At rates higher than this the flexion became more tonic and at 16 per second walking or stepping movements at about 1 per second were present. In a few instances where the voltage was increased to more than threshold, the stepping increased in amplitude and rate to about 4 per second as has been seen in other experiments with stronger shocks (Magoun, Ranson and Fisher, 1933). Stimulation in the more medial part of the internal capsule at rates above 21 per second frequently produced lapping movements. Lapping was never seen at rates slower than 12 per second and at that rate only at high voltages.

A moderate degree of facilitation was noted to repeated series of stimuli.

Effect of anesthetics on responses. The effects of nembutal were: Responses from stimulation of the motor cortex and internal capsule were present with an anesthetic dose of 15 to 20 mgm. of nembutal per kilogram but depressed or abolished by heavier doses.

Pupilloconstriction from stimulation of the posterior commissure was depressed as the dosage was increased.

Threshold for pupillodilatation from stimulation of the hypothalamus was not raised but was sometimes decreased by doses of nembutal as high as 45 mgm. per kilogram. The pupil became narrower after each additional injection (10 mgm. per kgm. at a time) until it was actually a slit. This probably had a bearing on the decreased threshold.

The response of the nictitating membrane from a stimulation of the hypothalamus was depressed by nembutal and frequently abolished. A typical experiment in which thresholds were determined under increasing depth of anesthesia showed that the thresholds to induction shocks at 12 per second rose as the dose per kilogram increased as follows: 15 mgm. per kgm., 7.0 volts; 25 mgm., 12.7 volts; 35 mgm., 40 volts; 45 mgm., no response.

Piloerection from stimulation of the hypothalamus was never obtained under nembutal.

The effects of urethane were: Responses from the internal capsule could not be obtained when the dose was 1.5 gram per kgm. In one animal flexion of the forelimb was obtained with induction shocks of 10 volts when the dose was 1 gram per kgm. but the threshold was raised to 28 volts by an additional 0.25 gram per kgm.

The effects of urethane on responses from the motor cortex were not studied.

Piloerection can be obtained under 1 to 1.25 gram urethane per kgm. from stimulation of the hypothalamus but is abolished by slightly increasing the dosage.

Pupillodilatation from stimulation of the hypothalamus is practically independent of the dose of urethane.

Retraction of the nictitating membrane is easily elicited under urethane in doses up to 1.5 gram per kgm. from stimulation of the hypothalamus but is greatly depressed by increased depth of anesthesia.

Effect of current type on the tissues. Faradic stimuli at the intensities used in these experiments do not affect the tissues, even after several minutes of stimulation, enough to alter the threshold or to alter the histological appearance as seen in sections stained by Weil or cresyl violet techniques.

Direct current stimuli when applied at low intensities for short periods of time through nichrome electrodes in many cases caused no detectable alteration in threshold or histological appearance. Occasionally the threshold would become abnormally high, due either to polarization of the electrodes, electrolytic damage to the tissues, or both, and there were small (0.5 mm. or less) but definite lesions produced. The lesions were apparent as evidenced by slight demyelination of fibers in Weil stains or

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Effect of anesthetics on responses. The effects of nembutal were: Responses from stimulation of the motor cortex and internal capsule were present with an anesthetic dose of 15 to 20 mgm. of nembutal per kilogram but depressed or abolished by heavier doses.

Pupilloconstriction from stimulation of the posterior commissure was depressed as the dosage was increased.

Threshold for pupillodilatation from stimulation of the hypothalamus was not raised but was sometimes decreased by doses of nembutal as high as 45 mgm. per kilogram. The pupil became narrower after each additional injection (10 mgm. per kgm. at a time) until it was actually a slit. This probably had a bearing on the decreased threshold.

The response of the nictitating membrane from a stimulation of the hypothalamus was depressed by nembutal and frequently abolished. A typical experiment in which thresholds were determined under increasing depth of anesthesia showed that the thresholds to induction shocks at 12 per second rose as the dose per kilogram increased as follows: 15 mgm. per kgm., 7.0 volts; 25 mgm., 12.7 volts; 35 mgm., 40 volts; 45 mgm., no response.

Piloerection from stimulation of the hypothalamus was never obtained under nembutal.

The effects of urethane were: Responses from the internal capsule could not be obtained when the dose was 1.5 gram per kgm. In one animal flexion of the forelimb was obtained with induction shocks of 10 volts when the dose was 1 gram per kgm. but the threshold was raised to 28 volts by an additional 0.25 gram per kgm.

The effects of urethane on responses from the motor cortex were not studied.

Piloerection can be obtained under 1 to 1.25 gram urethane per kgm. from stimulation of the hypothalamus but is abolished by slightly increasing the dosage.

Pupillodilatation from stimulation of the hypothalamus is practically independent of the dose of urethane.

Retraction of the nictitating membrane is easily elicited under urethane in doses up to 1.5 gram per kgm. from stimulation of the hypothalamus but is greatly depressed by increased depth of anesthesia.

Effect of current type on the tissues. Faradic stimuli at the intensities used in these experiments do not affect the tissues, even after several minutes of stimulation, enough to alter the threshold or to alter the histological appearance as seen in sections stained by Weil or cresyl violet techniques.

Direct current stimuli when applied at low intensities for short periods of time through nichrome electrodes in many cases caused no detectable alteration in threshold or histological appearance. Occasionally the threshold would become abnormally high, due either to polarization of the electrodes, electrolytic damage to the tissues, or both, and there were small (0.5 mm. or less) but definite lesions produced. The lesions were apparent as evidenced by slight demyelination of fibers in Weil stains or

failure of certain cells to stain properly with cresyl violet. Even more trouble was experienced with the modified direct current because of the greater intensities used. Less trouble was experienced with direct current after adoption of the method of silvering and chloriding the electrodes.

DISCUSSION. The thresholds were not determined for the cells and fibers stimulated but for a response of the corresponding effectors and would vary with "integration" in cranial and spinal centers. This is especially true when dealing with the cortex and internal capsule. Another site where the apparent threshold of a nervous structure might be modified is at the effector itself in the case of autonomic pathways. Rosenblueth's work (1932a, b) on autonomic pathways readily explains this statement.

The intensity of current required to stimulate nerve fibers in the central nervous system is high because of the shunting effect of inactive tissue. Where spatial facilitation is necessary to evoke a response the threshold may be higher if the pathway is diffuse rather than in compact fascicles. The threshold of the oculomotor nerve, stimulated in the brain stem was nearly 9 times that of the sciatic; a difference explained partly by difference in electrode type and partly due to the fact that the largest fibers of the sciatic may be a little larger than the largest fibers of the oculomotor, but mainly because of the shunting effect of the tissue of the midbrain.

Some doubt as to the desirability of faradic current for activating autonomic paths and centers has been raised by Hess (1932). He maintains that the threshold to faradic stimulation of autonomic structures is much higher than that of somatic structures and that the spread of the faradic current leads to erroneous conclusions because structures of low threshold at a distance from the electrodes may be stimulated, thus obscuring localization. On the basis of threshold experiments carried out on peripheral nerve, he suggests the use of a direct current interrupted 6 to 15 times per second, with the rate of rise and fall of current modified by condensers and choke coils.

There is no evidence to show that the threshold of a nerve fiber depends on the arbitrary divisions of the nervous system to which it belongs. On the contrary the threshold has been shown to depend on its conduction rate which in turn depends on fiber diameter (Blair and Erlanger, 1933). What Hess did in his experiments on peripheral nerve was to use a direct current of slow rise and fall which resulted in an increase in threshold of all fibers, but proportionally a greater increase in those of the somatic motor group. This caused the ratio of somatic threshold to visceral threshold to become more nearly unity. The reason for his results is not to be explained by some unknown property of the fiber depending on the structure it innervates, however, but rather due to the difference in average size of the nerve fibers in the nerves stimulated. For this reason conclu-

sions drawn from observations on a peripheral nerve are not necessarily valid for a part of the same functional system in the brain since the size of fibers in the central part of a pathway are not the same as that of the peripheral part.

The phenomenon underlying the degree of change of threshold with a certain degree of sloping of the current is that of "accommodation" as described by Hill (1936) and Solandt (1936). A nerve fiber possessing rapid accommodation will exhibit a more marked change of threshold to a current of slow rise or fall than one of slower accommodation. In considering the thresholds given in this paper it should be remembered that in order to duplicate the circuit constants of Hess, coils instead of resistances were used. The inductance of the coils is small but should not be neglected.

Stimulation with direct current has been successfully used by many workers on peripheral nerve. Rosenblueth (1932a) used rather brief rectangular shocks in his study of the autonomic nerves. However, for prolonged stimulation in the central nervous system where thresholds are necessarily higher, the possible destructive effect of direct current should be avoided.

The danger of decreased accuracy in localization due to spread of current is eliminated by thorough exploration of the regions under observation so that the most active points can be located. A response cannot be said to be due to spread of current if it cannot be elicited from any structure to which the current may have spread.

In view of the fact that a modified direct current has no demonstrable advantages over faradic current as a stimulating agent when used in the brain of an anesthetized animal, and because of the dangers of tissue destruction and polarization with continued use of direct current, there is no reason to encourage its use.

Whatever the type of current used for stimulation, spread to surrounding structures will occur if the intensity is increased enough, so that stimuli far in excess of the threshold values should not be used for localization work. The present data may give some idea as to the starting point of stimulus intensity in work of this type.

SUMMARY

Thresholds for responses of normal effectors were determined in the anesthetized cat for the hypothalamus, posterior commissure, oculomotor nerve, cervical sympathetic trunk, sciatic nerve, motor cortex and internal capsule. Induction shocks and direct current stimuli were used.

Some effects of nembutal and urethane on the responses are considered.

The effects of changes in frequency between 4 and 20 stimuli per second are given.

No advantages of direct current pulses with slow rate of rise and fall were discovered that justify its use in place of rectangular direct pulses or faradic shocks for work in the brain stem. Faradic stimuli, with the technique used in this laboratory, are adequate for accurate localization and have the advantage that there is less likelihood of polarization of electrodes or electrolytic damage to the tissues.

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NEUROMUSCULAR RESPONSES TO VARIATIONS IN CALCIUM AND POTASSIUM CONCENTRATIONS IN THE CEREBROSPINAL FLUID¹

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Although Sabbatini, in 1901, clearly demonstrated the importance of the calcium ion concentration on the activity of the cells of the central nervous system, the effect of the ionic balance in the cerebrospinal fluid on peripheral phenomena has been too often ignored since the first direct spinal puncture and injection reported by Corning in 1885. Weed pointed this out in 1919 in the course of a study of fluids suitable for use in the irrigation of the cerebrospinal canal. By comparing the gross effects produced by lowering the calcium of the blood serum (Hastings and Huggins, 1933) with those produced by the injection of sodium citrate in the cisterna (Huggins and Hastings, 1933), it could be inferred that the calcium ion concentration in the cerebrospinal fluid is of far greater importance for the production of peripheral neuromuscular symptoms than is the calcium ion concentration of the blood serum.

The present work was undertaken to investigate by means of sensitive recording devices the peripheral neuromuscular and circulatory responses to variations in calcium and potassium ion concentrations of the cerebrospinal fluid and to determine the relative importance of these ions in producing their peripheral effects.

METHOD. Thirty-six dogs anesthetized with sodium barbital or sodium pentobarbital (Nembutal) were used in these experiments. In order to change the concentration of calcium and potassium ions in the cerebrospinal fluid, a 16-gauge syringe needle with a special stylet was inserted into the cisterna magna. The stylet was withdrawn and a small rubber tube, one millimeter in external diameter, was injected through the needle from a syringe. The needle puncturing the cisterna was then removed, leaving the rubber tube in place (according to the technique described by Jacobs, 1933). Removal or injection of fluid from or into the cisterna

¹ A preliminary report of this work was presented at the meeting of the American Physiological Society at Detroit in April, 1935.

magna could then be carried out easily by the use of a syringe and a fine syringe-needle inserted into the exposed end of the rubber tube. This method has the advantage that it is possible to change repeatedly the contents of the cisterna without encountering blood if one so desires. (As many as 100 replacements of fluid have been successfully made in one experiment.)

Whenever changes were made or fluid was withdrawn from the cisterna, an equal volume (3 to 9 cc.) was immediately reinjected. This washing out process was usually immediately repeated two or three additional times with each experimental solution used. Samples of fluid removed from the cisterna were saved for calcium analysis. Occasionally samples of blood were simultaneously drawn and the serum analyzed for calcium.

Variations in muscle tension were measured in some cases by the use of a cathode ray oscillograph² and at other times by using a string galvanometer.³ The platinum needle lead-off electrodes were inserted either in the neck muscles or the upper foreleg or the thigh muscles of the animal. The results were recorded either by means of photographs of the cathode ray tube or by continuous photographic recordings of the movements of the string when the galvanometer was used.

In about half the experiments, blood pressure tracings were obtained, using a mercury manometer connected either with the common carotid or the femoral artery. When the blood pressure was recorded, respiratory movements were usually also recorded simultaneously.

Solutions used. A balanced isotonic salt solution, approximately the inorganic composition of cerebrospinal fluid, formed the basic solution of our experiments (solution 1). It contained 1.4 mM of calcium ions per liter and 6 mM of potassium ions per liter. By having a concentration of bicarbonate ions equal to 25 mM per liter and saturating the solution with 5.5 per cent carbon dioxide and 94.5 per cent oxygen, the pH of all solutions was maintained at approximately 7.4. Magnesium ions were present in a concentration of 0.5 mM per liter, and phosphates in a concentration of 1.0 mM per liter. The only variations made in the solutions used were in the concentrations of Ca^{++} and K^+ present. These, together with the appropriate solution number, are tabulated below:

	SOLUTION NUMBER				
	1	2	3	4	5
Ca^{++}	1.4	0	2.8	1.4	1.4
K^+	6.0	6.0	6.0	0	12

² We are indebted to Dr. W. H. Marshall for making the oscillographic records.

³ Dr. Edmund Jacobson kindly permitted us to use his highly sensitive string galvanometer.

When small amounts (0.5 cc.) of an isotonic solution of sodium citrate (77 mM per liter) were injected, this will be referred to as solution 6.

RESULTS. No change in peripheral muscle tension was observed in control experiments where 4 or 5 cc. of cerebrospinal fluid were withdrawn from the cisterna magna and then reinjected immediately. Neither was there any change in the state of activity of the musculature studied when a balanced salt solution (solution 1) was substituted for the cerebrospinal fluid of the dog (compare the first record of fig. 2 with the third

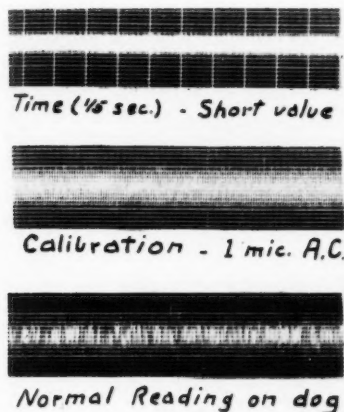


Fig. 1

Fig. 1. String galvanometer records of muscle tension and calibration of instrument. The short value indicates the string fluctuation with the amplification used. Calibration picture represents 1 microvolt A.C. superimposed on the short value (2.5 mm. deflection equals 1 microvolt). Normal reading on dog represents the state of muscular tonus in the animal before any cerebrospinal fluid was withdrawn. Muscle tension measured equals 4.4 mic.v.

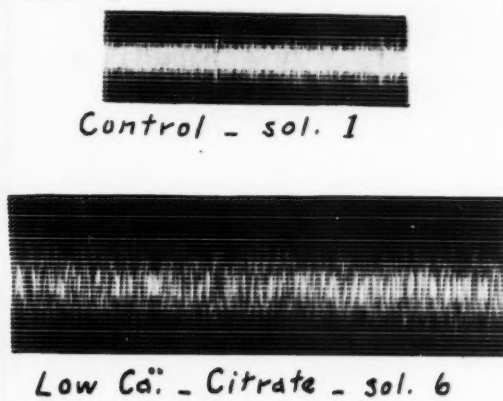


Fig. 2

Fig. 2. String galvanometer records of muscle tension. Control represents replacement of cisternal fluid with a balanced salt solution. Muscle tension equals 3.2 mic.v. Lowering the calcium ions by injection of 0.5 cc. sodium citrate into cisterna increases the muscular tension to 9.2 mic.v.

record of fig. 1). The washing out of the cisterna with a balanced salt solution or withdrawal and reinjection of cerebrospinal fluid had negligible effects on the blood pressure and respiration.

Effects of low calcium. When a solution identical in composition except for the fact that it contained no calcium (solution 2) was used to wash out and replace the cisternal fluid, there resulted a marked increase in muscular activity (see figs. 3 and 4) after the fourth or fifth replacement of fluid. The cerebrospinal fluid calcium was sometimes reduced to as low as 0.2 mM per liter by this procedure (see table 1). This effect came

on progressively, reaching its height a few minutes after the last replacement and lasting for about ten minutes. Only very slight changes were detected on the first washout of the cisterna, but they increased markedly in severity with succeeding replacements. The effects of increased muscular tension could easily be detected before any marked change in blood pressure or respiration was observed. When the changes of fluid were repeated several times in succession, a severe state of tetany supervened. The animal showed marked opisthotonos, pleurothotonos, and dorsal curvature of the tail, as well as extensor rigidity of the legs and contracted abdominal musculature. The neck and foreleg phenomena came on

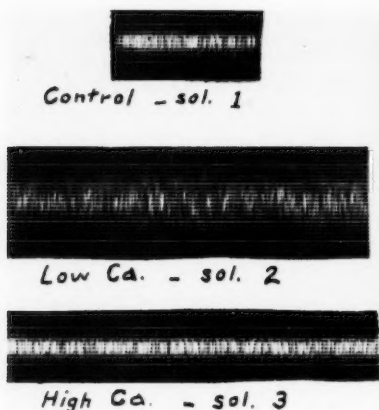


Fig. 3

Fig. 3. Galvanometer records of muscle tension when the concentration of calcium ions of the cerebrospinal fluid is varied. Control muscle tension equals 3.6 mic.v. Low calcium muscle tension equals 10.0 mic.v. Replacing low calcium with high calcium solution (solution 3) decreased muscle tension to 3.6 mic.v.

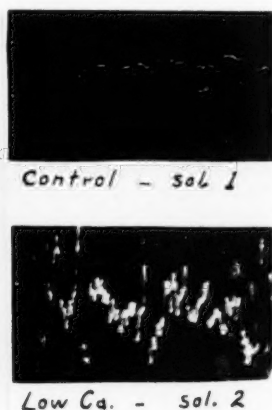


Fig. 4

Fig. 4. Cathode ray oscillograph records of muscle tension showing deviation from normal caused by reducing the calcium ion concentration of the cerebrospinal fluid, by washing out cisterna with a calcium-free solution.

first. Accompanying these symptoms, there was an elevation of blood pressure with a slowed heart. (After curare these circulatory effects were much less marked and the pronounced neuromuscular changes were abolished.) Respiration was markedly increased, and during severe attacks it became very dyspneic. The animal recovered from these attacks gradually, reaching a normal condition again in ten to fifteen minutes, depending upon the severity of the tetanic phenomena.

The lowered calcium content of the cerebrospinal fluid affected the circulatory centers in the brain, as evidenced by an alteration in the response obtained on stimulating the central end of one cut vagus nerve.

The normal pressor effects obtainable became more easily elicited while the depressor responses to appropriate stimulation were decreased. Apparently the reduction of calcium ions centrally augments pressor actions and inhibits depressor actions, so that a reversal of effect of stimulation of the central end of the vagus may occur (see Marshall, 1934).

The effects of low calcium were readily abolished in a minute or two by restoring the calcium ion concentration of the cerebrospinal fluid to normal (1.4 mM per liter) by using solution 3 (see fig. 3) with double the calcium content or even solution 1 (balanced salts), though the latter necessitated more replacements of fluid in the cisterna. (Table 1.)

Neuromuscular, circulatory, and respiratory phenomena identical with those just described could be elicited in an even more striking manner by the injection into the cisterna of 0.5 cc. of sodium citrate (77 mM per

TABLE 1

Peripheral effects compared with determined Ca^{++} concentration of cisternal fluid

Ca^{++} mM/L	EXPERIMENTAL SOLUTION USED	NEUROMUSCULAR CONDITION OF ANIMAL
1.45	Cisternal fluid	Quiet
1.56	Balanced ions	Quiet
0.35	Low calcium	Tetany
2.20	High calcium	Quiet
1.66	Balanced ions	Quiet
0.40	Low calcium	Tetany
0.21	Low calcium	Marked tetany
2.40	High calcium	Quiet
1.58	Balanced ions	Quiet
0.23	Low calcium	Marked tetany
1.62	Balanced ions	Quiet
1.54	Balanced ions	Quiet

liter). The action of citrate (solution 6) occurred very rapidly, starting ten seconds after its injection and reaching its maximum in a few minutes (see figs. 2 and 5). The effect was even more marked and lasted considerably longer than that produced by reducing the calcium ion concentration in the cisterna by washing out with a calcium-free solution.

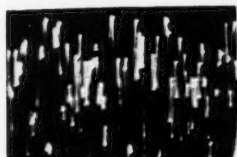
From the mass law equation for the ionization of calcium citrate (Hastings et al., 1934), it is possible to estimate the order of magnitude of the calcium ion concentration in the cerebrospinal fluid after the injection of the sodium citrate. Assuming that the volume of distribution of the citrate was in 10 cc. of cerebrospinal fluid, the Ca^{++} concentration would be approximately 0.1 mM per liter, an amount considerably less than was found by repeated replacement of the fluid with a calcium-free solution. This may account for the greater severity of the effects produced by citrate injections.

The citrate effect also could be overcome and the animal rapidly restored to normal by washing out with the high-calcium solution. Or the violently tetanic animal could be quieted by the replacement of 4 cc. of cisternal fluid by an isotonic magnesium chloride (107 mM per liter) solution.

Effects of high potassium. Changes in neuromuscular activity as well as circulatory and respiratory effects similar to but not so marked as those produced by reducing the calcium ions of the cerebrospinal fluid could be brought about by washing out the cisterna with solutions containing 12 mM of potassium per liter (solution 5). This effect was abolished by solutions deficient in potassium (solution 4) or those containing

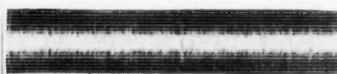


Control - sol. 1

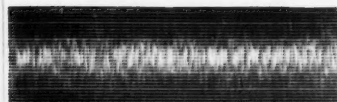


Low Ca^{++} - Citrate - sol. 6

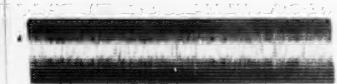
Fig. 5



Control - sol. 1



High K. - sol. 5



Low K. - sol. 4

Fig. 6

Fig. 5. Cathode ray oscillograph records of muscle tension showing deviation from normal caused by reducing the calcium ion concentration of the cerebrospinal fluid by injecting citrate into the cisterna.

Fig. 6. String galvanometer records of muscle tension. Control of 3.6 mic.v. increased to 8.8 mic.v. on increasing the potassium ion concentration in the cisterna. The lowest picture represents the return to a normal of 3.6 by washing with a potassium-free solution.

a normal concentration of potassium ions (see fig. 6). In this case also, the first neuromuscular phenomena occurred before there were any significant changes in blood pressure or respiration. But here also, the effects of ionic changes on the circulatory centers were brought out by changes in response to stimulation of the central end of one sectioned vagus nerve, similar to those discussed above after reduction of the calcium ion concentration. Here also, the effects produced increased with successive replacements. In this instance too, the pressor effects obtained were considerably reduced by curarization of the animal.

When the dorsal roots of the spinal cord in the lumbar region were

washed with solutions containing no calcium or high potassium, no neuromuscular changes were observed. Neither was there a noticeable change in muscular tension in the hind limbs when the exposed lower spinal cord was irrigated with these solutions. Application of the sodium citrate solutions to the appropriate region of the exposed cord did give rise to a muscular response in the hind limbs, however.

DISCUSSION. The results presented in the present paper support the view that widespread and marked neuromuscular, circulatory, and respiratory effects can be produced either simply by a decrease in the calcium ion concentration or an increase in the potassium ion concentration of the cerebrospinal fluid. These observations emphasize the importance of the ionic balance of the cerebrospinal fluid in determining the state of activity of the cells of the central nervous system. They furthermore suggest that the peripheral symptoms of tetany are to be referred, in large measure, to the calcium and potassium ion concentrations of the fluid bathing the cells of the higher centers. The great rapidity with which the results are brought about favors this idea, as does also the fact that in our experiments where blood was drawn during a tetanic seizure there was no change in blood serum calcium at that time. Experiments by Binger (1917) and by Storti (1935) who produced tetanic states and convulsive twitchings by the intravenous injections of phosphate and of oxalate were attended by a period of delay sufficiently long to have permitted the Ca^{++} of the extracellular fluids of the central nervous system to have been reduced in concentration. The clinical significance of disturbances of electrolytes in body fluids, especially in relation to uremia, is fully discussed in a recent review by Harrison and Mason (1937).

Our results and interpretations as to these ionic effects, although in the main the same, differ somewhat from those of Resnik and his coworkers (1936), in that the circulatory phenomena seemed more pronounced in their experiments than in ours. It seems to us that the marked circulatory and respiratory effects produced by lowering the calcium or increasing the potassium ions in the cerebrospinal fluid are in considerable part the result of the increased neuromuscular activity rather than predominantly or entirely central effects of the ions on cardiovascular centers in the region of the cisterna. The latter is undoubtedly one of the factors, however, as was shown long ago by Hooker (1915) in perfusion experiments using calcium and potassium. We were able to demonstrate marked increases in muscular tension at the onset of the convulsive state at a time before there were yet any significant changes in blood pressure or heart rate. Also, we could often obtain evidence of a very definitely increased neuromuscular state unaccompanied by circulatory variations. Furthermore, in our experiments the vascular effects were in part abolished by curare. In most of our experiments, the circulatory effects (slight rise

in blood pressure) were no greater than could be accounted for on the basis of the increased activity displayed by the animal. It is thought that part of the differences between the results of Resnik and coworkers and our own may, perhaps, be attributed to the greater concentrations of ions that they used and the slightly different method of administration of ionic solutions.

The degree of anesthesia undoubtedly plays a rôle in this type of response, because unanesthetized dogs showed more marked effects on the same amount of washing out of the cisterna with experimental solutions than did the anesthetized animals. We did not observe the depression following removal and reinjection of cerebrospinal fluid in an unanesthetized animal reported recently by Ivy and Schnedorf (1937), nor did washing the cisterna with a balanced salt solution produce a depressed state.

SUMMARY

1. Removing and reinjecting cerebrospinal fluid into the cisterna magna of anesthetized dogs has no effect on neuromuscular activity, nor have balanced salt solutions replacing cerebrospinal fluid in the cisterna any effect.

2. Lowering the calcium ion concentration of the cerebrospinal fluid, either by washing the cisterna with calcium-free solutions or injecting small amounts of sodium citrate into the cisterna causes the onset of a typical tetanic syndrome.

3. This seizure can be overcome and the animal returned to normal by restoring the calcium ions to their normal concentration.

4. Increasing the potassium concentration of the cerebrospinal fluid by replacing the fluid of the cisterna with a solution of increased potassium ion content also causes a marked increase in muscular tension of the voluntary muscles of the body. This can be overcome by restoring the normal potassium concentration of the cisternal fluid.

5. Associated with and apparently partially dependent on this marked increase in neuromuscular activity is a rise in blood pressure and increase in respiratory rate.

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EFFECT OF SYMPATHIN ON BLOOD SUGAR¹

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Cannon and Rosenblueth (1933,1935) have shown that sympathin, although like adrenine in action, is not identical with it. It seemed desirable to see whether it affects carbohydrate metabolism as adrenine does. Bacq (1933), indeed, observed a slight rise in blood sugar in two animals after stimulation of the lower abdominal sympathetic chains. But the small number of Bacq's experiments and the fact that Cannon and Rosenblueth (1933) by stimulating either the cardio-accelerator or the liver nerves obtained much more sympathin than was possible with Bacq's technique suggested a study of the effect of sympathin on blood sugar in a larger number of animals and with more favorable conditions.

Of the two rich sources of sympathin mentioned above, we had to use the cardio-accelerator nerves since, obviously, stimulation of the liver nerves would cause marked changes in the hepatic blood supply.

METHODS. Cats were used in this investigation. In order to stimulate the cardio-accelerator nerves without disturbing the blood-sugar level by involvement of the liver and the adrenal glands in an acute experiment, the adrenal glands had to be inactivated and the sympathetic innervation of the liver had to be destroyed in preliminary operations. This was done usually about two weeks before the actual experiment. The right adrenal was removed; the left was denervated by severing the left splanchnic nerves, excising that portion of the coeliac plexus in which they terminate, and removing the upper part of the left lumbar sympathetic chain (ganglia I, II and III). Also the left adrenal gland was demedullated by curettage after the medulla had been exposed through a lateral incision in the cortex. Sometimes the left adrenal was first denervated and demedullated and in a second operation the right adrenal was removed.

The destruction of the sympathetic innervation of the liver was carried out either by partial removal of the right sympathetic chain (from Th VII to L IV) in addition to the above operation on the left chain, or by severing the nerves in the hepatic pedicle and then painting it with con-

¹ Part of this work was presented in a paper read at the meeting of the Federation of the American Societies for Experimental Biology in Washington, D. C., April, 1936. Abstract appeared in *This Journal* 116: 12, 1936.

centrated phenol solution. In the former procedure, when the sympathetic supply to the liver is interrupted by section of the preganglionic neurons (decentralization), the sympathetic innervation of the liver is completely eliminated. In the latter, when the sympathetics are interrupted by section of the postganglionic neurons, the destruction of the liver sympathetics may not be quite complete, inasmuch as stripping the hepatic pedicle and painting it with phenol may not destroy possible sympathetic fibers in the media (Riegele, 1928); and if, as in man (Raigorodsky, 1928), sympathetic fibers from the phrenic plexus enter the liver near the posterior border, they are not destroyed. The latter technique also interrupts hepatic parasympathetic fibers. Later we shall refer to these animals as type I and type II animals. Type I and type II animals, then, are alike in that in both the right adrenal gland was removed, the left denervated and demedullated, but they differ in that the sympathetic supply to the liver was interrupted by section of the preganglionic neurons in type I and by section of the postganglionic neurons in type II (in which case the parasympathetic fibers were also severed). In type II animals the nictitating membrane was also denervated at the time of adrenal removal.

After the operation the animals were not used for experiments unless they were in perfect health and had regained their original body weight. On the other hand, they were used before regrowth of the severed nerves was probable. The animals were not fed for 24 hours before the experiment in order to exclude intestinal absorption.

In the actual experiment under anesthesia (see next section) and artificial respiration the stellate ganglia were exposed on both sides, the thoracic sympathetic chains interrupted, and the cervical sympathetic trunks and all the rami and branches sectioned. The internal branches of the right and left stellate ganglia (mainly the cardio-accelerator fibers) were then placed on shielded electrodes. For electrical stimulation a Harvard inductorium was used with 9 volts in the primary circuit. The strength of the stimulating current as regulated by the secondary coil distance will be given along with the experiments. Isotonic contractions of the nictitating membrane were recorded by a lever with a magnification of 14.

Blood samples for sugar determination were taken at stated intervals—the initial sample (before anesthesia) from the ear vein without exciting the animal, the later samples from the exposed femoral vein. The Hagedorn and Jensen (1923) method with a Somogyi (1930) filtrate was used.

Choice of anesthetic. An anesthetic suitable for our investigation must fulfill the following requirements: 1, it must not alter the blood-sugar level; 2, it must not interfere with the formation of sympathin; 3, it must not interfere with the local and remote effects of sympathin; 4, it must not prevent the hyperglycemic reaction caused by adrenine. This is a neces-

sary condition whether sympathin is expected to have an effect similar to adrenaline or not, since this responsiveness is the test of the reactivity of the preparation.

The first anesthetic tried was dial (Ciba), since Rosenblueth and Cannon (1932) found that it does not interfere with the liberation and effects of sympathin. Dial in the usual dose was injected into 3 normal and 3 type I cats, and the glycemic concentration determined every half hour for three hours. Since a marked hyperglycemia occurred in all these experiments, dial was discarded.

Griffith (1923) found in cats and Bodo and Neuwirth (1933) in dogs that chloralose does not appreciably affect the blood-sugar level. We tried both chloralose and chloralosane in doses from 0.08 to 0.10 to 0.12 grams per kgm. given as a 1 per cent solution intraperitoneally. Occasion-

TABLE 1

Effect of chloralose and chloralosane on the blood sugar of type I cats (adrenals inactivated; preganglionic section of liver sympathetics)

CAT NUMBER	BLOOD SUGAR IN MILLIGRAMS PER CENT												CHLORALOSE OR CHLORALOSANE INTRAPERITONE- ALLY
	Before chlo- ralosane	After chloralosane											
		1/2 hour	1 hour	1 1/2 hours	2 hours	2 1/2 hours	3 hours	3 1/2 hours	4 hours	4 1/2 hours	5 hours	5 1/2 hours	
4	74	69	70	74	73				73		75	74	0.1
16	72	74	69	69	70		70		68		70	70	0.1
6	71	72	72	75	74		72		70		70	74	0.12
7	68	83	74	68		66		70	68		68		0.12
5	81	90	84	79		80		80	79		81		0.12
8	69	82	75	70	72		68		69		71	70	0.11

ally vomiting and convulsive movements occurred. Usually the animal showed hyper-irritability when touched but became quiet in 20 to 25 minutes. In our experience the larger doses (0.10 to 0.12) were more satisfactory because the convulsions were lessened and the animals became quiet sooner. Of 6 normal cats (tested every half-hour for six hours) 3 showed practically no change of blood sugar; the other 3 showed a slight increase in the first half-hour; then the blood sugar returned to normal and remained for several hours at the original level. Similar results were obtained in type I cats (table 1). In cats 4, 16 and 6 the blood sugar remained practically unaltered for 6 hours, in cats 7, 5 and 8 after a transient rise during the first half-hour it returned to the original level. The initial rise was perhaps caused by the convulsions; since the glycemic level became constant later, the transient rise does not contraindicate the use of chloralose.

In order to test whether chloralose or chloralosane would prevent the hyperglycemic response to adrenalin, adrenalin in varying amounts was infused into a series of cats after a preliminary 2-hour period (table 2). It was given intravenously for 30 minutes in cats 9 and 10 at the constant rate of 0.00005 mgm. per kgm. per minute and in cats 11 and 13 at the rate of 0.000065 mgm. per kgm. per minute. Hyperglycemia resulted in all cases, averaging about 18 per cent. The rate of infusion was far below the upper physiological limits of reflex adrenine output (Cannon and Rapport, 1921). The rate corresponds to that given by Trendelenburg (1923) and Cori, Cori and Buchwald (1930) as being the minimal effective in raising blood sugar in unanesthetized normal rabbits. Chloralose and chloralosane certainly do not decrease appreciably the responsiveness to adrenalin.

TABLE 2

Effect of adrenalin on the blood sugar of type I cats (adrenals inactivated; preganglionic section of liver sympathetics). Chloralosane anesthesia

CAT NUMBER	BLOOD SUGAR IN MILLIGRAMS PER CENT								INTRA- VENOUS INFUSION OF ADRE- NALIN HCl
	Before chloral- osane	After chloralosane							
		½ hour	1 hour	1½ hours	2 hours		2½ hours	3 hours	
9	73	75	69	70	72	Adrenalin intra- venous in- fusion for 30 minutes	84	84	0.00005
10	81	81	82	82	80		90	92	0.00005
11	82	84	79	79	77		96	94	0.000065
13	69	72	72	68	70		88	91	0.000065

Does chloralose or chloralosane interfere with the liberation of sympathin or prevent the reactions of the effectors to sympathin? To answer this question, animals were used in which the adrenals had been previously inactivated and the nictitating membrane chronically denervated. Under these anesthetics stimulation of either the cardio-accelerator or the liver nerves caused contraction of the nictitating membrane, and in the former case acceleration of the heart. The drugs do not interfere, therefore, with the production of sympathin nor with its local and remote effects. These, then, were the anesthetics used in later experiments.

Effects of electrical stimulation of the cardio-accelerator nerves in operated animals. For stimulation the secondary coil of the inductorium was so adjusted that a decided increase in heart rate occurred without spread of the current, as indicated by no contraction of the neighboring muscles. The coil distance at first was usually 12 to 11 cm. and later was gradually decreased in order to maintain a fairly uniform acceleration. In our

earlier experiments with type I animals the right and left cardio-accelerator nerves were alternately stimulated, each nerve receiving 3 ten-minute periods of stimulation—not continuous, but interrupted five times a minute by means of a mechanical interrupter.

In three of the eight experiments no change at all was observed; in the other five there was a rise which varied from 14 to 22 mgm. per cent and

TABLE 3

Blood-sugar changes in type I animals (adrenals inactivated; preganglionic section of liver sympathetics) produced by simultaneous electrical stimulation of right and left cardio-accelerator nerves

Stimulation: Ten one-minute periods at intervals of 5 to 8 minutes

CAT NUMBER	BLOOD SUGAR IN MILLIGRAMS PER CENT														
	0 hour		4 hour		1 hour		1 1/2 hours	2 hours	2 1/2 hours	3 hours	3 1/2 hours	4 hours	4 1/2 hours	5 hours	5 1/2 hours
22	71	Chloralosane 0.10 to 0.12 gm./kgm. intraperitoneally. Artificial respiration started	72	Isolation of right cardio-accelerator nerve	74	Isolation of left cardio-accelerator nerve	75	74*	73	79	94		99	101	
								xxxxxxx							
12	71		72		72		72	75*	74	79	90		88	90	
								xxxxxxx							
26	78		93		93			86*	84	83	78	86	117	131	
								xxxxxxxxxxxxxxxxxxx							
15	79		82		85			82*	72	76		87		97	99
								xxxxxxxxxxxxxxxxxxx							
23	87		103		101			92*		92		102		123	127
								xxxxxxxxxxxxxxxxxxx						143	
19	74		84		78			75*		73		85	116	129	
								xxxxxxxxxxxxxxxxxxx							
27	79		78		78			79*		76		90	119	124	
								xxxxxxxxxxxxxxxxxxx							

xxxxxxx indicates electrical stimulation.

* Sample taken just before stimulation started.

averaged 17.6 mgm. per cent. In view of the varying results, it appeared desirable to change the mode of stimulation.

The most satisfactory results were obtained when the right and left cardio-accelerator nerves were simultaneously stimulated 10 times for 1-minute periods. The 1-minute stimulation periods were continuous, no interrupter being used. Time was allowed to elapse between stimulations in order to permit the heart to regain its original rhythm. This type of stimulation was adopted in all our final experiments.

In table 3 are the results obtained in type I animals. In every case there was a rise in blood sugar. There was very little change during the time (60 to 90 minutes) of the 10 electrical stimulations plus the intervening rest periods. Sometimes at the end of this period there was a slight rise, as in cats 23, 19, 27, but usually the effects became more visible about $\frac{1}{2}$ to 1 hour after the stimulations ceased. Thereafter the blood-sugar level rose steadily (except in cat 12) and for 2 hours did not show any decline. The rises in blood sugar were higher than those in the positive earlier experiments, ranging from 15 to 54 mgm. per cent and averaging 36 mgm. per cent.

The heart rate always increased at the start of each stimulation and usually returned to normal after the stimulation ceased. Although 5 to 8 minutes were usually sufficient for full recovery, longer rest periods were occasionally necessary. The heart did not always respond to successive stimulations with the same acceleration unless the coil distance was decreased, and sometimes the original increase was not obtained even when the stimulus was increased until muscular contraction occurred from spread of current. Since Dye (1935) found a relative inexhaustibility of sympathin stores it seems probable that this diminished local effect of sympathin may have been due to "polar fatigue," for we did not shift the electrodes to fresh portions of the nerves.

These results indicate that the hyperglycemia was caused by sympathin produced in excessive quantities by the stimulation of the cardio-accelerator nerves. To prove, however, that cardiac sympathin, besides having a local action, diffused into the blood stream and was carried to remote organs in quantities sufficient to be effective, we used the chronically denervated nictitating membrane as an indicator. And that we might obtain a sensitized liver and thus a quicker and greater effect on the blood-sugar level, we interrupted the hepatic sympathetic supply at the post-ganglionic neurons.

Table 4 presents the results obtained in type II animals. Cardio-accelerator stimulation again caused a rise in all experiments, but the rise appeared much sooner, usually within half an hour from the start. The hyperglycemia was very marked, the increases sometimes amounting to 100 or 200 mgm. per cent and reaching a much earlier maximum. Cats 17 and 18 showed a slight hyperglycemia even before the electrical stimulation started, a fact to which we shall refer later. In cat 18 chloralose also caused a slight rise.

In these experiments the strength of current was so adjusted as to produce not only increases in heart rate but also marked contractions of the chronically denervated nictitating membrane. To obtain these effects stronger currents were necessary than in the experiments in table 3, but still no contraction of neighboring muscles was seen. Sufficient time was allowed between stimulations for the nictitating membrane to relax and

the heart rate to return to normal. In order to produce the same effects at successive stimulations the current often had to be increased, but sometimes even this partially failed.

Comparison of the results obtained in type I and type II animals reveals that much greater increases in blood sugar appeared in the latter. This may be accounted for by the following factors: 1, stronger currents were used (greater quantity of sympathin produced); 2, the sympathetic nerves

TABLE 4

Blood-sugar changes in type II animals (adrenals inactivated; postganglionic section of liver sympathetics; nictitating membrane denervated) produced by simultaneous electrical stimulation of right and left cardio-accelerator nerves

Stimulation: Ten one-minute periods at intervals of 5 to 8 minutes

CAT NUMBER	BLOOD SUGAR IN MILLIGRAMS PER CENT																	
	0 hour		1/2 hour		1 hour		1 1/2 hours	2 hours	2 1/2 hours	3 hours	3 1/2 hours	4 hours	4 1/2 hours	5 hours	5 1/2 hours	6 hours		
14	79	Chlorlosane 0.10 to 0.11 gm./kgm. intra-peritoneally. Artificial respiration started	82	Isolation of right cardio-accelerator nerve	80	Isolation of left cardio-accelerator nerve	83*	101	140		155		143		145			
							XXXXXXX											
17	88		92		94		105*	118	311		251		173					
							XXXXXXX											
18**	75		88		84		98*	171		195	200		194	189	193	194		
							XXXXXXXXXXXXX						XX	XX	XX			
32	63		69		66		68*	116		122		112		109				
							XXXXXXXXXXX											
36	76		78		76		82*	128		127		94		90				
							XXXXXXXXXXX											
29	80		85		81		83*		128		156	157		128		111		
							XXXXXXXXXXXXX											

xxxxxxx indicates electrical stimulation.

* Sample taken just before stimulation started.

** Cat 18 received in addition to the regular ten one-minute periods of stimulation three one-minute periods of stimulation between the hours 4 1/2 to 6.

to the liver were interrupted by section of the postganglionic neurons, and the blood-sugar results suggest that this more peripheral interruption of the liver nerves may sensitize the liver to adrenaline or sympathin. The slight hyperglycemia observed in cats 17 and 18 before electrical stimulation may have been due to the sensitization and to mechanical stimulation of the cardio-accelerator nerves while being prepared. 3, As stated above, animals were not used unless they had regained their original body weight

and were in good health. However, the type II animals were exceptionally well nourished, having gained 20 to 30 per cent over their original weight, and probably had rich glycogen stores in the liver. Since the hyperglycemic response to adrenine is related to the amount of hepatic glycogen, a rich reserve probably played a part in the hyperglycemia following the discharge of sympathin.

In excluding other agents than sympathin as the cause of the hyperglycemia, several possibilities have to be considered. Adrenine as a possible cause can be excluded, since in our animals the right adrenal was removed and the left was not only denervated but also demedullated. In all of the type II animals histological examinations were made of the left adrenal gland. Of a total of 17 animals, all showing similar experimental results, only those in which complete absence of the medulla was histologically demonstrated are presented in table 4 and in table 5. The adrenal glands were fixed in Zenker's fluid and later embedded in paraffine. From 6 to 12 sections were cut from different levels and stained with hematoxylin and eosin. The cortical tissue in every instance showed little change. The cortical cells were abundant and well preserved, although the cords of cells in the fascicular zone were sometimes disarranged by scars where it had been traversed by the curette. The lipid content of the cortical cells was greatly diminished, but in a few focal areas just internal to the glomerular zone groups of cells were strikingly swollen with lipid material in fine droplet form. In the region of the medulla the results of the curette were obvious. The medullary tissue was completely absent. In its place were large dense fibrous scars, some of which had become extensively calcified. Others contained deposits of cholesterol crystals surrounded by foreign-body giant cells and hemosiderin pigment-granules contained within phagocytes.²

The possibility that muscle metabolites, especially lactic acid, might cause the hyperglycemia was also examined. Although we took special precautions to prevent spread of current, we wished nevertheless to learn the effect of powerful muscular contractions. In type II animals the cardio-accelerator nerves were prepared just as before but instead of these nerves the pectoral muscles were stimulated; and blood sugar, heart rate and nictitating membrane were observed. As shown in table 5, a preliminary slight rise in blood sugar followed the administration of the anesthetic and the preparation of the stellate ganglia. There was, however, no further rise either during the electrical stimulation or throughout the course of the experiment; on the contrary, in cat 33 there was a slight drop. The heart rate did not increase, the sensitized nictitating membrane did not contract and

² We take this opportunity to express our indebtedness to Dr. S. L. Wilens and Mr. L. Nadvorney, Department of Pathology, New York University College of Medicine, who made the histological examinations for us.

the pupils remained small. These results exclude any possible rôle of muscle metabolites in these experiments.

We conclude then from our experiments that sympathin produced by electrical stimulation of the cardio-accelerator nerves is the cause of the observed hyperglycemia.

It will be noted that the sympathin hyperglycemia outlasted the other sympathin effects by several hours. This is little indication of how long liberated sympathin persists without destruction, for adrenine hyperglycemia similarly outlasts other adrenine effects for considerable lengths of time though it is known to be destroyed rapidly.

TABLE 5

Blood-sugar changes in type II animals (adrenals inactivated; postganglionic section of liver sympathetics; nictitating membrane denervated) produced by simultaneous electrical stimulation of right and left pectoral muscles

Stimulation: Ten one-minute periods at intervals of 5 to 8 minutes

BLOOD SUGAR IN MILLIGRAMS PER CENT																	
CAT NUMBER	0 hour	Chloralose 0.10 gm./kgm. intra-peritoneally. Artificial respiration started	1/2 hour	Isolation of right cardio-accelerator nerve	1 hour	Isolation of left cardio-accelerator nerve	1 1/2 hours	2 hours	2 1/2 hours	3 hours	3 1/2 hours	4 hours	4 1/2 hours	5 hours	5 1/2 hours	6 hours	
25	80		90		94		92*		94	93			92		91		91
									XXXXXXXXXXXXX								
33	84		99		101		103*		101	96			90		84		79
									XXXXXXXXXXXXX								
24	72	74	72	72*		73	73			70		68		70			
						XXXXXXXXXXXXX											

xxxxxxx indicates electrical stimulation.

* Sample taken just before stimulation started.

Whether sympathin hyperglycemia is caused only by mobilization of liver glycogen or whether in addition sympathin may interfere with the utilization of carbohydrates in a manner similar to adrenine, we are unable to discuss. Whether sympathin has any other adrenine-like effects on carbohydrate metabolism (decrease in muscle glycogen, increase in blood lactic acid), we are planning to investigate later.

We do not wish to draw inferences from these experiments as regards the physiological rôle of sympathin in blood-sugar regulation, since the stimuli necessary to produce sympathin in effective quantities were far from physiological.

In a forthcoming paper we shall present the results obtained with sympathin produced by emotional excitement without anesthesia.

SUMMARY

1. Chloralose and chloralosane are suitable anesthetics for the study of the effect of sympathin on blood sugar because *a*, they leave the blood-sugar level uniform for many hours (sometimes after a slight preliminary hyperglycemia) (see table 1), *b*, they do not interfere with the liberation and the effects (local and remote) of sympathin, and *c*, they do not prevent the typical hyperglycemic effect of adrenine (see table 2).

2. Dial is not suitable for this purpose because it causes a marked hyperglycemia.

3. In animals with adrenal glands inactivated and liver sympathetic nerves destroyed, sympathin produced by electrical stimulation of the cardio-accelerator nerves causes hyperglycemia (see tables 3 and 4).

4. The degree of the hyperglycemia depends *a*, upon the quantity of sympathin (which in turn is determined by the type, intensity and duration of stimulation), *b*, upon the condition of the liver which if sensitized will respond with a greater glycogen breakdown, and *c*, upon the glycogen content of the liver.

5. Electrical stimulation of large groups of muscles does not change the blood-sugar level (see table 5).

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HYPERGLYCEMIA PRODUCED BY SYMPATHIN IN EMOTIONAL EXCITEMENT¹

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In previous work it was shown that sympathin produced by electrical stimulation of the cardio-accelerator nerves caused a rise in blood sugar in adrenal-inactivated and liver-denervated animals (Bodo and Benaglia, 1936, 1938). Since in these experiments an anesthetic and strong electrical stimuli had to be used, we thought it desirable to study the effect of sympathin produced under conditions free from an anesthetic. If sympathin produced by emotional excitement in an unanesthetized animal would cause hyperglycemia, it would throw further light on the question whether sympathin has a rôle in normal blood-sugar regulation.

METHODS. Cats were used exclusively in this work. In one group in preliminary operations the adrenals were inactivated. This consisted of removal of the right adrenal gland, and denervation and demedullation of the left in a manner described in a previous paper (Bodo and Benaglia, 1938). In addition the liver was also denervated by severing the nerves in the hepatic pedicle and then painting the latter with a concentrated phenol solution. The completeness of this technique was critically discussed in the paper referred to above. The nictitating membrane was also denervated by extirpating the right superior cervical ganglion. As before, the animals were not used unless they were in perfect health, having not only regained their original body weight but increased it 20 to 30 per cent. At this time they undoubtedly had a high percentage of liver glycogen. If they were used in successive experiments care was taken to allow a sufficiently long interval between experiments for recovery of glycogen stores (8 to 10 days).

Emotional excitement was induced in two ways. In the first they were excited with aggressive barking dogs for varying lengths of time (3 to 30 minutes), in the second they were made to struggle on a cat board from 5 to 15 minutes. The former we term emotional excitement not accom-

¹ Part of this work was presented in a paper read at the meeting of the Federation of the American Societies for Experimental Biology in Washington, D. C., April, 1936. Abstract appeared in this Journal **116**: 12, 1936.

panied by struggle and the latter emotional excitement accompanied by struggle.

The animals were not fed for 24 hours previous to the actual experiment. Samples of blood for sugar determination were taken from the marginal vein of the ear before the experiment and at regular intervals after stimulation. The Hagedorn and Jensen method (1923) with a Somogyi filtrate (1930) was used.

1. *Effect of emotional excitement not accompanied by struggle.* This was studied in 16 normal and 24 operated animals. Each animal was tested on at least two different occasions. In confirmation of Britton (1928), a few minutes of stimulation was found to be sufficient to bring about hyperglycemia in every normal animal. Within 2 to 3 minutes from the end of a 3 to 10 minute stimulation we observed a rise of 23 to 43 mgm. per cent averaging 35 mgm. per cent (32 experiments). The maximum appeared either at this time or within the next 30 minutes. The blood sugar then returned gradually to normal. Great differences were noted in the degree of hyperglycemia. These cannot be attributed to differences in liver glycogen since well fed animals such as these, though they show variations in their liver glycogen, always contain a considerable amount. Even fasted animals with an initial liver glycogen content of only 1 per cent still respond to adrenaline with a hyperglycemia not materially different from that observed in the well fed animals (Bodo, Benaglia and Friedman, 1933, 1938). Similar results were obtained by Kuriyama (1918) and Markowitz (1925). We think that the differences in the excitability of the animals and to some extent the duration of the excitement are the factors causing the variations in hyperglycemia.

Of the 98 experiments performed on 24 adrenal-inactivated and liver-denervated cats, we present in table 1 only the experiments on those cats in which it has been histologically demonstrated that no traces of adrenal medulla were present (for histological description see paper by Bodo and Benaglia, 1938). The other animals gave quite similar experimental results, but are not presented since in them were found traces of the medulla.

As before, each animal was tested on two or three occasions always allowing time for glycogen recovery. Only 5 of the 12 cats in table 1 (cats 17, 14, 24, 2, 21) showed a rise in blood sugar on every occasion when excited with barking dogs. Cats 25, 18, 34, 33 were consistently negative in that the blood-sugar level remained constant even when the stimulation period was prolonged for 30 minutes. Cats 32 and 29 showed no rise on one occasion but did on another. Cat 36 gave negative results in two of three experiments.

The positive experiments in this group were similar to those of the normal animals insofar as the rise in blood sugar was noticeable in the sample taken usually 2 to 3 minutes after the end of the stimulation, the

TABLE 1

Effect of emotional excitement not accompanied by struggle on the blood sugar of adrenal-inactivated, liver-denervated cats

CAT NO.	NUMBER AND DATE OF EXPERIMENT	BLOOD SUGAR IN MILLIGRAMS PER CENT						DURATION OF EXCITEMENT, MINUTES	SUMMARY OF BLOOD-SUGAR CHANGES*
		Before excitement	After excitement						
			3 min.	23 min.	48 min.	78 min.	108 min.		
25	1. 5/17/35	82	81	81	81	82	82	5	—
	2. 6/ 4/35	83	80	83	86	84	84	30	—
18	1. 11/ 4/35	77	80	80	77	78	79	15	—
	2. 11/22/35	80	79	77	80	80	83	30	—
34	1. 5/17/35	76	76	79	78	76	76	5	—
	2. 6/ 4/35	76	77	78	77	77	77	30	—
33	1. 2/ 8/35	82	80	84	78	82	84	5	—
	2. 2/27/35	85	80	84	81	83	84	30	—
32	1. 11/ 2/35	74	74	78	75	75	79	10	—
	2. 11/19/35	76	91	86	86	83	80	10	+
29	1. 11/11/35	82	93	89	91	91	89	15	+
	2. 11/28/35	81	75	75	75	75	70	15	—
36	1. 9/27/35	74	74	73	71	71	73	15	—
	2. 10/ 5/35	78	78	71	70	78	78	15	—
	3. 10/14/35	85	101	96	94	93	90	10	+
17	1. 10/ 5/35	76	90	89	83	83	80	10	+
	2. 10/22/35	72	87	85	81	80	77	30	+
14	1. 9/26/35	78	95	95	91	86	83	15	+
	2. 10/15/35	74	89	87	84	81	78	10	+
24	1. 9/27/35	74	93	90	89	83	81	30	+
	2. 10/14/35	83	92	93	89	88	85	15	+
2	1. 5/17/35	82	99	93	91	89	83	10	+
	2. 6/ 4/35	78	89	87	85	84	84	15	+
21	1. 2/ 8/35	77	87	87	83	82	82	10	+
	2. 2/27/35	74	85	86	82	80	79	15	+

* + indicates an increase in the blood-sugar level.

— indicates no change in the blood-sugar level.

maximum appearing at a corresponding time, followed by a gradual return to normal. The rise in blood sugar, however, was much less than in normals; it varied from 10 to 20 mgm. per cent and averaged 15 mgm.

per cent. As stated above, these animals were in excellent condition and the differences in the degree of hyperglycemia observed in these and in normal animals can be attributed not to lack of glycogen but to lack of adrenaline. The animals in the operated group, which always failed to respond to barking dogs, were inexcitable; perhaps they had been raised near dogs and were accustomed to their barking.

II. *Effect of emotional excitement accompanied by struggle.* Animals that had been used in the previous series were now subjected to excitement produced by struggle. In all of the normal animals this type of excitement, lasting 5 to 10 minutes, caused a much greater and more prolonged rise than excitement brought about only with barking dogs. The rise amounted to 55 to 120 mgm. per cent, averaging 79 mgm. per cent (32 experiments); the maximum again appearing at a time corresponding to that of previous experiments.

The operated animals listed in table 2 were the same animals that had been subjected to barking dogs (table 1) and had been shown to have no adrenal medulla. If we compare table 1 with table 2, it can be seen that all the cats which gave a positive response when excited with barking dogs (cats 17, 14, 24, 2, 21) responded similarly with hyperglycemia when they struggled on the board. But in addition cats 34, 33, 32, 29, 36 also showed a rise in blood sugar on every occasion when struggling, in contrast to the entirely negative or only occasionally positive results obtained with the emotional excitement without struggle. Cats 25 and 18 gave consistently negative results in both types of experiments. The rise of blood sugar in the positive experiments varied from 10 to 36 mgm. per cent and averaged 16 mgm. per cent. Compared with table 1 the difference is not so much in the degree of hyperglycemia as in the number of positive experiments. In our experiments we found a constant though not linear relation between the duration of stimulation and the degree of hyperglycemia.

We believe that the cause of the hyperglycemia in the above experiments on adrenal-inactivated, liver-denervated cats was sympathin, produced in excess at the sympathetic effectors by emotional excitement and then carried to the liver in effective quantities. Contraction of the chronically denervated nictitating membrane, which was noted, was another indication of the presence of sympathin in the blood stream. If sympathin is truly the cause, a completely sympathectomized animal should show no change in blood sugar after excitement by struggle. This was conclusively verified experimentally in the following ways. We observed completely sympathectomized cats during struggle unintentionally induced by ordinary gentle laboratory handling (passing of a stomach tube, tying to a board) go into epileptiform convulsions. The blood sugar during this period did not show any changes. Furthermore, struggle has been observed by Bodo, CoTui and Benaglia (1936, 1937, 1938) in completely sympathec-

TABLE 2
*Effect of emotional excitement accompanied by struggle on the blood sugar of
 adrenal-inactivated, liver-denervated cats*

CAT NO.	NUMBER AND DATE OF EXPERI- MENT	BLOOD SUGAR IN MILLIGRAMS PER CENT						DURATION OF EXCITEMENT, MINUTES	SUMMARY OF BLOOD- SUGAR CHANGES*
		Before excite- ment	After excitement						
			3 min.	23 min.	48 min.	78 min.	108 min.		
25	1. 5/24/35	80	78	78	82	78	78	5	—
	2. 6/12/35	78	75	78	77	81	81	15	—
18	1. 11/14/35	80	79	77	80	80	83	10	—
	2. 12/ 2/35	76	78	78	80	76	77	15	—
34	1. 5/24/35	76	112	101	91	84	75	5	+
	2. 6/12/35	78	108	98	84	85	82	15	+
33	1. 2/18/35	85	95	95	94	93	88	5	+
	2. 3/ 8/35	82	92	86	82	80	79	12	+
32	1. 11/11/35	76	91	86	86	83	75	5	+
	2. 11/28/35	79	89	88	85	82	78	10	+
29	1. 11/20/35	80	91	90	87	85	85	5	+
	2. 12/ 9/35	77	90	88	85	83	75	10	+
36	1. 10/21/35	81	97	91	90	86	79	5	+
	2. 10/29/35	75	90	91	82	80	81	10	+
17	1. 10/14/35	73	87	88	82	78	76	5	+
	2. 10/30/35	70	84	80	78	72	73	10	+
14	1. 10/ 4/35	72	94	94	88	82	79	5	+
	2. 10/23/35	69	87	84	80	74	68	10	+
24	1. 10/ 7/35	77	91	92	87	86	80	5	+
	2. 10/23/35	85	96	95	92	87	82	10	+
2	1. 5/24/35	75	88	86	83	79	76	5	+
	2. 6/12/35	78	94	91	87	83	83	10	+
21	1. 2/18/35	75	90	88	86	82	79	5	+
	2. 3/ 8/35	72	87	86	84	78	77	10	+

* + indicates an increase in the blood-sugar level.

— indicates no change in the blood-sugar level.

tomized cats following the administration of morphine. The blood sugar, however, showed a distinct drop rather than a rise, whereas before complete removal of the sympathetic chains the adrenal-inactivated cats showed a hyperglycemia during the excitement induced by morphine.

In both instances the blood lactate must have been high, which is evidence that lactic acid, undoubtedly also present in the blood in large concentration in the emotional excitement experiments, was not the cause of the hyperglycemia. Anoxemia undoubtedly present during the violent muscular exercise accompanying struggle, as evidenced by the panting, could not have had any hyperglycemic effect through liberation of adrenaline (Cannon and Hoskins, 1911; Zwemer and Newton, 1928), since only those animals are presented in which the histological examination testified to the complete absence of medullary tissue. Again it failed to cause hyperglycemia in the completely sympathectomized animals and so it could not have exercised a direct action on the liver cells.

Cats (normal or operated) exposed for the same period to an aggressive barking dog or made to struggle for equal periods with approximately equal intensity exhibit varying degrees of emotional excitement and accompanying hyperglycemia. These vary even in the same animals on different occasions. Since liver-glycogen variations can be neglected as an important cause of the varying degrees of hyperglycemia for the reasons stated before, it appears that the quantity of sympathin (quantity of sympathin plus adrenaline in normal animals) produced under identical conditions in different animals and even in the same animal must vary a great deal. This is the explanation we offer for the different degrees of hyperglycemia obtained in different animals and also for the occasional negative results described above. We believe from our experiments that struggle produced a larger quantity of sympathin than excitement due to barking dogs.

The foregoing experiments in conjunction with those of Bodo and Benaglia (1938) undoubtedly showed that sympathin—whether produced by electrical stimulation of the two cardio-accelerator nerves or by emotional excitement—can produce hyperglycemia in adrenal-inactivated and liver-denervated animals when circulating in the blood stream in sufficient concentration. Before discussing whether or not sympathin actually has a rôle in the carbohydrate metabolism of normal animals under physiological conditions, we feel obliged to consider an apparent paradox. In the electrical stimulation experiments only two nerves were stimulated (right and left cardio-accelerators) and the strength of current was adjusted as to obtain a decided contraction of the chronically denervated nictitating membrane. These animals were under chloralose or chloral-osane anesthesia and the contractions of the membrane were recorded. In the experiments reported in the present paper the entire sympathetic nervous system was stimulated without the use of an anesthetic. Contractions of the chronically denervated nictitating membrane were observed but not recorded. Striking differences were seen in the degree of hyperglycemia. In the electrical stimulation experiments it sometimes amounted to 100 to 200 mgm. per cent, whereas in the emotional excite-

ment experiments it averaged 16 mgm. per cent and never exceeded 36 mgm. per cent. We are unable to offer a definite explanation for this rather unexpected phenomenon.

On first thought one would expect a greater quantity of sympathin to be produced by stimulation of the entire sympathetic nervous system than by stimulation of only 2 nerves; but one could imagine that the powerful electrical stimuli applied to the cardio-accelerators could be sufficiently intense to produce the larger quantity of sympathin. Though the nictitating membrane was the indicator in both types of experiments, the contractions were recorded graphically in the anesthetized animals and only observed in the unanesthetized. It can not, therefore, be used for the purpose of a quantitative comparison between the two types of experiments.

The presence of an anesthetic in one group may be another factor responsible for these discrepancies. It is conceivable that the glycogen-mobilizing effect of sympathin is affected to a lesser degree by chloralose than the actions at other sympathetic effectors (nictitating membrane). The anesthetic may also influence other regulatory mechanisms; for instance, it might interfere with the secretion of insulin.

We can not attribute the differences in our results to a difference in feeding. Cannon and Griffith (1922) have shown that a high protein diet favors hepatic sympathin production but this can not be a factor in our results since in a large number of cases the same animals were used in both types of experiments. They were first used on several occasions in the emotional excitement experiments (tables 1 and 2 of this paper) and later in the electrical stimulation experiments (table 4 in the previous paper) and were kept on the same diet throughout.

On the basis of our experimental results we think that sympathin has a definite rôle in the normal carbohydrate metabolism but we are not prepared to state to what extent sympathin is responsible for glycogen mobilization under normal conditions.

Two factors which would tend to modify normal function claim our attention. First, in order to be able to study the effect of the circulating sympathin on the liver, the latter organ had to be denervated. In the earlier paper (Bodo and Benaglia, 1938) we presented evidence that this procedure may sensitize the liver to the glycogen-mobilizing effect of sympathin in a manner analogous to the sensitization of other sympathetic effectors. This factor would tend to increase the effect of sympathin on blood sugar. Second, the adrenals had to be inactivated so that the effect of sympathin on blood sugar would not be masked. In view of the work of Rosenblueth and Cannon (1932), Liu (1935) and Rosenblueth and Morrison (1934) the combined effects of adrenaline and sympathin on the denervated nictitating membrane are greater, under certain conditions, than the sum of the individual effects. In other words, there can exist between adrenaline and

sympathin not only an additive but a synergistic or potentiated effect. It is not unreasonable to assume that the glycogen-mobilizing effect of sympathin can also be potentiated by adrenine. If this is the case, our experiments would not reveal the effect of sympathin in the presence of adrenine in a normal animal. In the light of these two opposing influences, one tending to lessen, the other to exaggerate the effect of sympathin on blood sugar, there seems to be no way of deciding from our experiments what the exact rôle of circulating sympathin in the normal regulation of blood sugar may be.

In this connection the question arises, to what extent do the sympathetic nerves to the liver play a part in glycogen mobilization. In other words, to what extent does sympathin produced locally in the cells by nerve stimulation (intercellular nerve endings having been described by Riegele, 1928) play a part in glycogen mobilization? Most investigators attribute the main rôle in glycogen mobilization to adrenine. According to Griffith (1923), Bulatao and Cannon (1925) and Britton (1928) the liver sympathetics play only a minor part. The liver, then, seems to occupy a unique position in its differing reaction to adrenine and sympathin. Adrenine has a powerful effect on glycogen mobilization, whereas sympathin produced locally would have only a minor effect. The conditions are certainly entirely different from those in all other sympathetic effectors.

SUMMARY

1. Emotional excitement unaccompanied by struggle (lasting from 3 to 30 minutes) causes either no change or only a slight rise in the blood-sugar level in adrenal-inactivated and liver-denervated cats (see table 1) in contrast to the marked hyperglycemia observed in normal cats with a much shorter period of excitement (3 to 10 minutes).

2. Emotional excitement accompanied by struggle causes in normal cats a much greater and more prolonged hyperglycemia than the excitement without struggle.

3. Emotional excitement accompanied by struggle causes either a slight hyperglycemia or none at all in the adrenal-inactivated, liver-denervated cats. A larger number of these animals responded with hyperglycemia to this type of excitement than to the excitement without struggle (see tables 2 and 1).

4. Completely sympathectomized animals show either no change or a slight fall in the blood-sugar level during struggle.

5. The cause of the slight hyperglycemia obtained in the adrenal-inactivated, liver-denervated cats is sympathin produced by excitement at the sympathetic effectors and carried to the liver as well as to other regions, as manifested by the contraction of the chronically denervated nictitating membrane.

6. The degree of the hyperglycemia in all cases depends upon the excitability of the animal and the duration of the excitement (quantity of sympathin in the adrenal-inactivated cats), upon the condition of the liver (degree of sensitization) and to a limited degree upon the glycogen content of the liver.

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THE PANTING RESPONSE OF NORMAL UNANESTHETIZED DOGS TO MEASURED DOSAGES OF DIATHERMY HEAT

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Animals such as the dog, cat and rabbit, which have a deficiency of sweat glands, when heated develop a type of respiration called "panting," "heat polypnea" or "heat tachypnea." When panting commences there is a distinct change in the type of respiration which is characterized by a marked increase in rate and a reduction of tidal volume. Quantitative measurements on the changes which occur in respiration before and after panting are few. This is due possibly to the mechanical difficulties involved where the respiratory rate is high. With rates of 200 to 300 per minute the errors of moving mechanical systems due to starting inertia are so great that customary methods of measuring ventilation rate and tidal volume are useless for quantitative measurements. The few investigations made on this problem have been made on anesthetized animals in which tracheal cannulas were used. Anesthetics produce varying degrees of respiratory depression and seriously disturb the temperature regulating mechanism. In addition to the difficulty encountered in matching the dead space a tracheal cannula used to study panting has a more serious objection. In normal panting the respired air is blown over the moist surfaces of tongue, mouth, and pharynx from which evaporation for cooling purposes takes place. When a tracheal cannula is used the respired air does not pass over these moist surfaces but through the cannula. A cannula thus deprives an animal of a considerable part of the evaporating surface. Since there are no data on the changes in the type of respiration of normal unanesthetized dogs breathing in a normal manner without masks or cannulas the following experiments were performed. Attention has been directed particularly to the changes of respiration before the onset of panting and the type of breathing which results from continued heating.

METHODS AND PROCEDURE. To measure ventilation rate and tidal volume a body plethysmograph of the Haldane (1935) type was used. A trained dog lay in a closed plethysmograph with head protruding through a

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seal at the neck. Low inertia valves permitted air to pass through the plethysmograph during respiration. The outlet valve discharged into a 150 liter closed tank. To the outlet tube from this tank there was connected a small aluminum spirometer and a recording gas meter. When air was discharged from the plethysmograph into the tank the increase in pressure was indicated on the spirometer. Suction was then applied through the gas meter until the spirometer returned to its base line. The

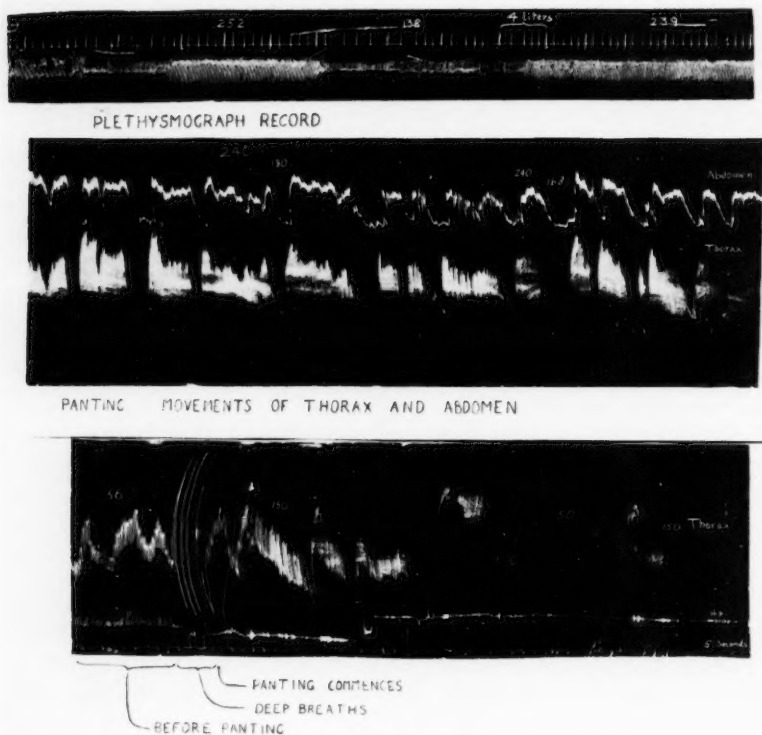


Fig. 1

large air volume, i.e., 150 liters, behaved as a pneumatic cushion, and a continuous pressure increase was produced rather than a pulsating pressure at high respiratory rates.²

Respiratory rates were measured by a small tambour whose natural frequency exceeded the highest respiratory rate. A kymograph record of the recording gas meter (top tracing), the respiratory rate (middle tracing) and a 5 second signal magnet (lowest tracing) is shown in figure 1.

² Details of this plethysmograph to be published in "Science."

In some experiments where changes in respiration rate and body movement were to be determined a thread was stretched over the thorax, abdomen and hip of a quiet, recumbent dog. These threads were attached to levers for kymographic recording. The two lower tracings of figure 1 were obtained by this method.

In order to obtain uniform and reproducible conditions of heating, the plethysmograph and room were maintained at 30-31°C. with a relative humidity of 50 ± 10 per cent. The dogs were heated by diathermy current from a machine described by Hemingway and Witts (1936). Using this method the amount of heat received by the dogs could be computed from the high frequency voltage and current, as described by Hemingway and McClendon (1933). The heating rates were made equal to and twice

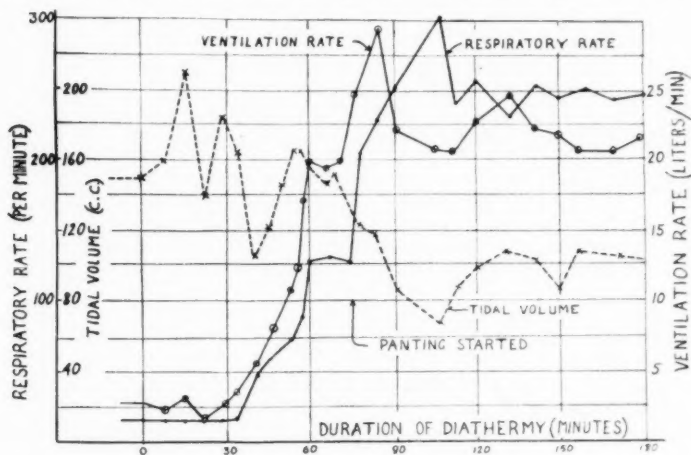


Fig. 2

the b. m. r., computed from the metabolism determinations of Kitchen (1923), and the treatment continued for 3 hours. The dogs rested two hours in the plethysmograph before being heated. The temperatures were measured by thermocouples.

RESULTS. Ten experiments were performed on 5 dogs, each dog being heated at a rate of 1.0 and 2.0 b.m.r. units on different days. Figure 2 shows how the tidal volume, ventilation rate and respiratory rate vary during a 3 hour diathermy treatment. This graph is typical but each dog has individual peculiarities of respiratory response. For convenience the respiratory response has been divided into the following parts: the prepaning interval, the onset of panting and the period of panting. The prepaning interval can be divided into two subdivisions which may be designated as part I, wherein the low rates of the resting period are main-

tained or become even lower, and part II where the respiratory rate progressively increases until panting starts. Figure 3 may be considered as an atypical response since part II of the preanting interval is absent. The onset of panting in this case was very sudden, there being no preliminary rise of rate before panting commenced. This animal has a respiratory response similar to Anrep's vagotomized dogs.

Table 1 contains the summarized data taken from the 10 respiration-time curves. The *basal* values are those at the beginning of the heat treatment. The minimum values of the respiratory rate under basal conditions at 30 to 31°C. and 40 to 60 per cent humidity are 8 to 40 per minute. This rate is comparatively low compared to the panting values. The ventilation volume is low and for dog A, which was especially cooperative in his

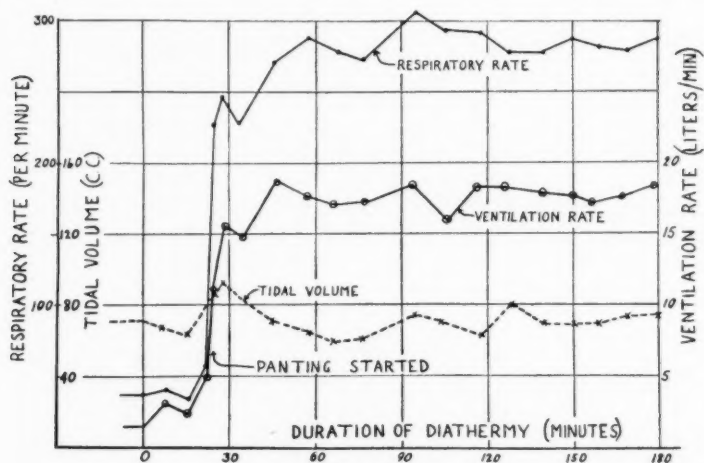


Fig. 3

ability to relax, the ventilation rate was 1.6 liters per minute. During *part I of the preanting period* the basal values are continued or in some cases the respiration rates become less. During this interval the dog is drowsy and apparently quite comfortable. Such a response resembles that of patients undergoing diathermy treatments who often remark on a feeling of comfort and drowsiness during diathermy treatments. At the beginning of *part II of the preanting period* the respiratory rate and ventilation rate commence to rise. There is a progressive increase in both rates until panting starts. During this interval the average increase in ventilation rate is 3.5 fold while the respiratory rate increase is 3.2 fold. The increase of ventilation rate results from an increase in respiratory rate, the tidal volume on the average remaining unchanged. In contrast

with part I of the prepaning period the dog exhibits symptoms of restlessness and discomfort. Untrained or partially trained animals will attempt to stand or struggle. These symptoms of discomfort continue throughout this interval but disappear when panting starts.

The onset of panting is marked by a respiratory rate increase of 2.7 times over the highest prepaning rate while the tidal volume is reduced 25 per cent on the average. An object of this investigation was to determine if there is an abrupt change in respiration when panting starts or if there is a merging of the prepaning type into panting by a continuous increase of respiratory rate. The results show that within a few seconds there can

TABLE 1

DOG	HEAT DOSAGE = 1.0 B.M.R. FOR 3 HOURS					HEAT DOSAGE = 2.0 B.M.R. FOR 3 HOURS				
	A	B	C	D	E	A	B	C	D	E
Weight of dogs (kilos).....	17.2	19.1	18.0	17.5	12.1					
B.M.R. (per dog) watts.....	38.1	42.4	40.0	38.7	26.8					
Basal rectal temp.....	37.7	37.9	38.2	37.3	38.4	38.0	37.6	38.0	37.5	37.9
Rectal temp. increase.....	1.15	0.95	0.3	0.9	0	0.85	1.45	1.0	1.3	0.9
Humidity.....	50	48	55	57	50	50	48	40	60	48
Basal resp. rate.....	19	39	46	37	34	29	17	14	71	8
Basal tidal volume.....	72	156	119	124	73	66	149	161	80	103
Basal vent. rate.....	1.4	6.1	5.8	4.6	2.6	1.9	2.6	2.2	6.1	0.82
Final prepaning resp. rate.....	58	111	151*	98	87	56	126	176	106	105
Final prepaning tidal vol.....	83	172	91*	118	78	71	157	196	58	86
Final prepaning vent. rate.....	4.9	19.1	12.7*	11.4	7.0	3.9	19.8	17.9	6.2	8.4
Initial panting resp. rate.....	230	204	222	250	236	204	336	263	270	
Initial panting tidal vol.....	83	140		66	59	58	125	56	58	57
Initial panting vent. rate.....	11.0	21.9		15.4	13.8	12.1	24.8	19.6	12.1	13.7
Average panting resp. rate.....	277	251		256	275	285	246	360	320	293
Average panting tidal vol.....	74	113		72	52	55	101	50	48	51
Average inter-panting resp. rate.....		145		164	109		155	228		125
Average inter-panting tidal vol.....		150		96	83		125	89		75
Average vent. rate after on- set of panting.....	16.6	22.7		16.6	9.7	14.9	22.8	17.8	14.6	12.3

* Rapid respiration without panting. These are average values for the period remaining after maximum respiration and ventilation rate were reached.

be an abrupt increase in respiration rate and that a condition of panting can be diagnosed from respiration rate alone. This is illustrated in the middle kymograph tracing of figure 1, which shows thoracic movements at the onset of panting. It is to be noted in this tracing that the prepaning type of respiration was labored in that the whole body moved as indicated by the record of hip movement of the recumbent dog. When panting started this labored type of breathing disappeared.

After panting has once commenced and while the animal continues to be heated the type of respiration resembles that of the lowest tracing of figure 1. There are short intervals of true panting alternating with periods

of a type of breathing resembling the prepanting type which is designated as an interpanting. During the interpanting interval the rate is relatively low and the tidal volume high. During the panting intervals the respiratory rate varied from 200 to 350 respirations per minute and the tidal volume decreased as the rate increased.

With a heat dosage equal to the b.m.r. dog C did not pant but maintained a high respiratory rate of about 150 once this value was reached. With dog A there was no appreciable respiration during the interpanting interval. This interval became a momentary pause.

DISCUSSION. It was formerly believed as a result of the experiments of Uyeno (1923) that during panting the breathing becomes very shallow with tidal volumes being reduced to about one-sixth to one-eighth of the basal values. This finding of Uyeno was believed to add support to the theory of panting see (Bazett, 1927), that a shallow rapid respiration favored evaporation by air movement over the moist surfaces of mouth, nose and pharynx without creating too great a stirring of the alveolar air with loss of carbon dioxide. Anrep and Hammouda (1932) using anesthetized dogs and tracheal cannulas in experiments similar to Uyeno's, did not find such low tidal volumes during panting but found a reduction of tidal volume to about 50 per cent of the basal values. Anrep and Hammouda on repeating Uyeno's results identically found that the low tidal volumes of Uyeno were due to the use of Muller valves which did not respond to the rapid pressure changes of panting. Our results on tidal volume changes agree in general with the results of Anrep and Hammouda and lead to the conclusion that very shallow breathing in panting does not occur.

There is, however, another observation which lends support to the theory that panting is adopted to prevent too great a disturbance of alveolar air while increasing the fanning movement of air through the mouth. The disturbance of alveolar air is determined by the ratio of tidal air, ΔV , to total air in the lungs, V . A low ratio of $\Delta V/V$ would tend to minimize the disturbance of alveolar air and this ratio would be lowered by a low tidal volume ΔV . Another method of reducing this ratio would be to increase V . From records of abdominal and thoracic movements as shown in the middle tracing of figure 1 it is to be noted that panting consists of a thoracic movement superimposed on a state of abdominal inspiration. In other words at the outset of a panting interval the lungs are filled with air by abdominal inspiration and this is maintained throughout the interval of thoracic panting. At the end of the panting interval there is abdominal expiration which continues throughout the interpanting interval. Hence during panting V is increased and the ratio $\Delta V/V$ is decreased partially by reducing ΔV and also by increasing V . This state of inspiration during panting was also observed on records made by connecting a sensitive spirometer directly to the closed plethysmograph. A

kymograph record from such a spirometer, while not responding quantitatively to the rapid panting rate, does show the slower respiration on which the rapid breathing is superimposed.

It is of interest in determining the effect of anesthetics on the panting response to compare the results here reported with those of Anrep and Hammouda (1932) who used chloralose-urethane anesthesia. These authors designate as "mild panting" a respiratory rate of 120 to 150 per minute and a tidal volume reduced from a basal value of 42 ml. to a panting value of 25 ml. They call "severe panting" the respiratory movements when the rate reaches 200-350 per minute, but with these high rates the tidal volume *increased* from a basal of 42 ml. to values of 80 to 90 ml. With our anesthetized dogs there was no panting with respiratory rates less than 200. Only when panting started at rates of 200 to 350 per minute was the tidal volume reduced. In none of the experiments did we observe an increase of tidal volume of the magnitude described by Anrep and Hammouda. It seems that the urethane chloralose anesthesia 1, depresses the ability to lower tidal volume with the high respiratory rates, and 2, lowers the respiratory rate at which panting starts.

Doubling the heat dosage did not appreciably change the respiration response. One would expect increased ventilation and possibly respiratory rate with an increased heat dosage. No such change was found.

It is a pleasure to acknowledge the interest, coöperation and assistance of Dr. H. G. Barbour, who followed the course of this problem in detail. We wish to thank Dr. R. W. Clark for useful suggestions and the loan of apparatus.

SUMMARY

Trained normal dogs have been heated with diathermy, receiving heat dosages equal to, and double the b. r. at 30 to 31° room temperature and with a humidity of 40 to 60 per cent. The respiratory rate, tidal volumes and ventilation rates have been measured with a recording body plethysmograph similar to that of Haldane, and with a meter system for measuring ventilation rate designed in a special way for high respiratory rates.

When an animal is heated by diathermy, the usual response is a gradually increasing ventilation and respiratory rate with no appreciable change in tidal volume. When panting occurs, there is an abrupt increase of two to three times in respiratory rate over the rate observed immediately before panting. The ventilation rate increases to almost double the final pre-panting value. Continued heating results in only a slight increase of respiratory and ventilation rates over the initial panting values. When panting occurs, the tidal volume is reduced 20 to 40 per cent. From kymograph records of chest movement and a plethysmograph it has been observed that a dog pants by thoracic movements during a state of inspira-

tion maintained by the abdominal muscles. Panting with the lungs filled with air would tend to reduce the disturbance of alveolar air resulting in a lowered CO_2 tension.

Comparing these results with those of Anrep and Hammouda (1932) who used urethane-chloralose anesthesia, it has been shown that this type of anesthesia does not permit the lowered tidal volume with the high respiratory rates of 200 to 350 per minute as occurs in normal dogs without anesthesia and which, according to theory, is desirable to prevent loss of carbon dioxide by hyperventilation.

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THE EFFECTS OF HYPOPHYSECTOMY AND OF ANTERIOR PITUITARY EXTRACTS ON THE DISPOSITION OF FED CARBOHYDRATE IN RATS¹

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When hypophysectomized rats are fasted, they suffer abnormal losses of body carbohydrate, especially of muscle glycogen (1). They also appear to oxidize more carbohydrate during this time than do normal animals with the same amounts of carbohydrate available at the start of the fasting periods (2). Anterior pituitary extracts have been shown to restore both the fasting glycogen levels and the respiratory quotients to the normal fasting levels (2, 3, 4). The question then arises whether the defect in the carbohydrate metabolism in hypophysectomized rats—an apparent difficulty in repressing carbohydrate oxidation—is demonstrable only in the fasted state; or whether it can be shown to affect the disposition of fed carbohydrate. Also, it becomes desirable to determine the action of anterior pituitary extracts in fed as well as in fasted hypophysectomized rats, and also, if possible, to demonstrate such action in normal rats.

A few papers bearing on these points have appeared. Fisher and Pencharz reported in a short note that hypophysectomized rats oxidized a given amount of fed glucose in about the same time as did normal rats, but that since their oxygen consumption was much less, they appeared to derive a correspondingly greater proportion of their total energy from carbohydrate (5). These authors did not otherwise determine the fate of the fed glucose, so that its proportionate disposition in the absence of the hypophysis was unknown. After the work reported in this paper had been partially completed, a preliminary paper appeared by Myer, Wade and Cori (6), in which was reported the action of an anterior pituitary extract on the disposition of carbohydrate fed to normal animals. Insofar as this work was duplicated by that presented here, the results are in substantial agreement. No reports have appeared concerning the effects of anterior pituitary extracts on the metabolism of hypophysectomized rats fed glucose, or on normal fasted rats.

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² Porter Fellow, 1937-38.

PROCEDURE. The experiments reported here were performed as follows: Normal and hypophysectomized rats were fasted 24 and 18 hours respectively, and then fed known amounts of glucose by stomach tube. The oxygen consumption, respiratory quotients and nitrogen excretion were then determined during the next several hours. Then the animals were killed and the levels of blood glucose and of muscle and liver glycogen were determined, as were also the amounts of glucose remaining in the alimentary tracts. Identical experiments were performed at the same times on normal and hypophysectomized rats which had been treated for various lengths of time with anterior pituitary extracts. Respiratory data and carbohydrate levels were also determined in normal fasted rats, with and without previous anterior pituitary injections. The respiratory quotients of unfasted normal and hypophysectomized rats were also studied.

All the animals used were young male rats, weighing from 150 to 180 grams at the time of the experiments. The operations for removal of the hypophyses were performed by the parapharyngeal route; their completeness was determined by search of the sella turcica at autopsy in all cases, and also by lack of gain in body weight and by the atrophic condition of the adrenals and testes. The operated rats were used for these experiments from 1 to 3 months after the operation. Previous to the experiments, the animals were all fed a stock diet high in carbohydrate, as described in (1).

The glucose fed was prepared in 25 to 40 per cent solution. In each case, 5 cc. of the solution was fed by stomach tube without anesthesia. The exact amounts of glucose fed were determined by analyses of aliquot samples of the solutions used for feeding.

The details of the determinations of carbohydrate were the same as those described in (1). All samples were taken under amytal anesthesia. For the determination of the amounts of glucose absorbed, the alimentary tracts of the rats were put into hot water immediately after their removal, cut up, and extracted repeatedly with warm water. The extracts were treated with West's mercuric sulfate reagent, copper tungstate or zinc hydroxide (Somogyi) for the removal of non-fermentable reducing substances. The action of these agents was checked by fermentation tests. Nitrogen determinations were made by the micro-Kjeldahl method.

The apparatus used for determining the respiratory metabolism was a seven-unit machine designed by Dr. Max Kleiber. In it, the oxygen consumption was measured by replacement with water in calibrated cylinders, the oxygen being under a constant small positive pressure. Carbon dioxide production was determined by titration in the absorbing batteries charged with solutions of KOH in an excess of BaCl_2 . The temperature in the apparatus was kept at 27°C . The usual period over which oxygen consumption was measured was 3 hours, after omission of the time taken

for attainment of equilibrium in the respiratory apparatus. The calculation of the non-protein respiratory quotients and of the amounts of carbohydrate oxidized were made by the usual methods. Average figures only for the nitrogen excretion were used, because of the individual variation encountered when the collection periods were so short. As these amounts were never very large, errors due to this procedure were not great. The figures for oxidation of carbohydrate were calculated on the basis of total time elapsed between the feeding of the glucose and the taking of the tissue samples, usually a little more than 4 hours.

TABLE 1

Carbohydrate oxidation and deposition 4 hours after feeding glucose to fasted rats

	NUMBER OF RATS	GLUCOSE ABSORBED	O ₂ CONSUMPTION	R.Q.	R.Q. NON-PROTEIN	BLOOD GLUCOSE	LIVER GLYCOGEN	MUSCLE GLYCOGEN	NITROGEN EXCRETION
		mgm. per 100 grams	cc. per 100 grams per hr.			mgm. per cent	mgm. per 100 grams body wt.	mgm. per cent	mgm. per 100 grams per hr.
A. Normal rats, unfed									
1. Untreated	15		154±1.1	0.724±0.003		72	1.2	524	
2. A.P. injections*	12		150±1.4	0.722±0.003		65	1.9	570	
B. Normal rats fed glucose									
1. Untreated	10	854	147±2.1	0.857±0.005	0.862	132	123	762	1.9
2. A.P. injections									
Over 3 days*	9	789	167±2.9	0.784±0.007	0.782	142	106	885	2.1
Once only†	3	700	170	0.763	0.760	131	124	843	2.3
3. A.P. injections chronic‡	6	810	137±2.4	0.868±0.008	0.880	118	107	762	3.0
C. Hypophysectomized rats fed glucose									
1. Untreated	8	568	106±3.0	0.909±0.008	0.925	79	28	520	2.2
2. A.P. injections†	8	457	99±3.8	0.812±0.008	0.814	156	34	502	0.7

* One cubic centimeter standard alkaline extract per day 3 days; 0.5 cc. 1 to 3 hours before glucose was fed.

† One cubic centimeter standard alkaline extract 1 to 3 hours before glucose was fed.

‡ One cubic centimeter standard alkaline extract per day 20 days; 1 cc. 2 hours before glucose was fed.

The anterior pituitary extract used was one of the standard alkaline extracts of beef described previously (3), containing about 14 mgm. per cubic centimeter of organic material. It contained all the known anterior lobe hormones, and was very potent in preventing the fall in muscle glycogen levels which occur in fasted hypophysectomized rats (M.E.D. less than 1 mgm. per 100 grams) (4). Injections were given intraperitoneally, 1 to 3 hours before the glucose was fed; also, in some of the normal rats, as described below, daily injections were made during 3 days and during 20 days preceding the actual experiments.

RESULTS. The averages of the figures obtained in the eight series of experiments on animals fasted or fed glucose are presented in table 1.

Approximate figures for the probable error of the averages have been included where most important. The other figures were all averages of series in which the individual values agreed fairly closely. In tables 2 and 3 are presented figures calculated from those in table 1, showing the proportionate disposition of the fed carbohydrate and its relation to the total energy metabolism. The results are conveniently discussed as follows:

1. *Effects of hypophysectomy.* That the removal of the pituitary affected the metabolism of fed carbohydrate as well as of body carbohydrate in fasted animals is well shown in these tables. The untreated hypophysectomized rats when fed glucose developed higher respiratory quotients

TABLE 2
Disposition of fed carbohydrate in 4 hours after feeding
Glucose in milligrams per 100 grams

	OXIDIZED	LIVER GLY- COGEN	FOUND AS BLOOD AND TISSUE GLU- COSE	MUS- CLE GLY- COGEN	TOTAL
Untreated normal rats					
1. Mgm. CH ₂ O* per 100 grams.....	453	122	30	120	724
Per cent of absorbed CH ₂ O.....	53 ± 1.5	14	4	14	86
2. Injected with A.P. extracts					
a. Acute treatment mgm. CH ₂ O.....	243	104	40	158	545
Per cent of absorbed CH ₂ O.....	31 ± 2.5	13	5	20	70
b. Chronic treatment mgm. CH ₂ O.....	406	106	20	120	652
Per cent of absorbed CH ₂ O.....	50 ± 1.8	13	3	15	81
Hypophysectomized rats					
1. Untreated					
Mgm CH ₂ O.....	420	28	11	85	544
Per cent of absorbed CH ₂ O.....	74 ± 3.7	5	2	15	96
2. Injected with A.P. extracts					
Mgm. CH ₂ O.....	201	33	50	75	359
Per cent of absorbed CH ₂ O.....	44 ± 3.7	7	11	16	78

* Carbohydrate as glucose.

than did the normal rats. Much less glycogen was deposited in the liver of these animals, and the blood sugar levels, too, were low. More striking differences were shown to exist when the actual disposition of the fed carbohydrate was calculated, as shown in table 2. The hypophysectomized rats appear to have oxidized 74 per cent of the absorbed glucose as compared to 53 per cent in the normal series. The figures for deposition of carbohydrate in tissues and blood were calculated by comparing the levels found with those usual in unfed hypophysectomized rats (350 mgm. per cent muscle glycogen, 55 mgm. per cent blood glucose, and 0.5 mgm. per 100 grams body weight as liver glycogen after fasting 18 hours); it was

assumed that the blood glucose and muscle glycogen were distributed in 50 per cent of the body weight. From this comparison, it is seen that the hypophysectomized rats deposited only 5 per cent of the absorbed glucose as liver glycogen, when the normal proportion was 14 per cent. The glucose in the tissues of the hypophysectomized rats also appeared to be a smaller proportion of the absorbed amount than in the normal animals. However, the proportion of the absorbed glucose deposited as muscle glycogen was about the same as in the normal, probably in part because of the low initial glycogen levels in the fasted operated rats (1).

The fact that hypophysectomized rats deposit less of their absorbed glucose as glycogen was found in earlier experiments by Bennett (7). Now it would seem that the increased oxidation of the fed carbohydrate which occurs in these animals largely accounts for this difference.

Although the proportions of the absorbed glucose metabolized showed the changes described above, the actual quantity of carbohydrate apparently oxidized in the two series was about the same. This was the case in spite of the fact that the total oxygen consumption (and the rate of absorption of the fed glucose) was much smaller in the hypophysectomized animals. Therefore, as shown in table 3, and as previously stated by Fisher and Pencharz, the proportion of the total energy obtained from carbohydrate must have been greater in the operated rats.

2. *The effects of anterior pituitary extracts in acute experiments.* As seen in table 1, the most noticeable effect of the anterior pituitary extracts in the rats fed glucose was the immediate reduction of the respiratory quotients. In the hypophysectomized animals, these figures were brought down to well below the normal. The blood sugar levels were increased markedly, but there was little change in the liver or muscle glycogen. In the normal rats, also, a marked effect of the extract on the respiratory quotients was observed; and although the blood and liver carbohydrate levels were little different from those in untreated rats, the muscle glycogen values were much increased, in some cases to over 1000 mgm. per cent. The absorption of glucose and the total oxygen consumption of the hypophysectomized rats were not improved by the treatment.

The injection of the anterior lobe extract more than compensated for the removal of the hypophysis in affecting the disposition of the fed carbohydrate. The proportion of the absorbed glucose which was oxidized was reduced to below the normal; and also, as shown in table 3, the proportion of total energy obtained from carbohydrate by the hypophysectomized rats was affected similarly. The proportion of the glucose deposited as liver glycogen was not greatly affected, but instead there was a large increase in the amount of blood and tissue glucose. Reduced liver function in the hypophysectomized rats may have contributed to this effect.

In the fed normal animals treated with the extract in these acute exper-

iments, the results were similar to those in the hypophysectomized animals. In this series, the proportion of absorbed glucose oxidized was also much diminished; and although there was little change in the liver glycogen or tissue glucose distribution, the proportion deposited as muscle glycogen was greatly increased. An unexplained change was observed in the oxygen consumption of these normal animals, when they were injected with the A.P. extract. No change in this value was observed in the unfed rats, but in those fed glucose, an increase of 16 per cent occurred in the oxygen uptake. However, no such change was found in the fed hypophysectomized rats.

The results of anterior lobe treatment of normal unfed rats are also included in table 1. As seen there, no changes were observed in the respiratory figures, and there was only a slight increase in muscle glycogen

TABLE 3
Energy metabolism during 4 hours after feeding glucose
(Calories ($\times 1000$) per 100 grams per hour)

	TOTAL CALORIES	CALORIES FROM CARBOHYDRATE	
		Calories	Per cent of total calories
Normal rats			
1. Untreated.....	717	369	52
2. Acute treatment			
(a) Treated over 3 days.....	800	201	25
(b) Treated once only.....	807	144	18
3. Chronic treatment.....	672	355	53
Hypophysectomized rats			
1. Untreated.....	525	345	66
2. Injected with A.P. extracts.....	473	182	39

($+46 \pm 11$ mgm. per cent). These figures stand in contrast to those obtained in fasted hypophysectomized rats (2). However, they need not be surprising, since possibly the oxidation of carbohydrate was already at such a low level in the normal fasted rats as to make it difficult to produce a further decrease in it. The fact that no change in the R.Q. was produced by the injections of the extracts showed that the changes observed in the other series were not due to alterations in acid base balance or to other non-specific effects.

3. *The effects of chronic treatment with A.P. extracts.* It was found recently in this laboratory by Mr. L. L. Bennett that after chronic treatment with A.P. extracts, hypophysectomized rats became refractory to the action of these extracts in maintaining fasting muscle glycogen levels (8). In view of this finding, it seemed advisable to try chronic treatment in conjunction with the experiments described above.

As noted before, the effect of the anterior lobe extract in these experiments was an immediate one, being evident in a few hours. Rats injected for three days prior to the final experiment also responded in the same way as those previously untreated. However, when normal rats were pre-treated for 20 days with the usual alkaline extract, there was absolutely no effect of the final dose of the extract, given just before the experiments. Evidently, in these normal rats, a refractory state had developed toward the A.P. factor responsible for the depression in carbohydrate oxidation after glucose feeding. This effect would appear quite analogous to that obtained in fasting hypophysectomized rats on prolonged treatment with A.P. extracts.

One more point deserves mention concerning the figures presented in table 2. For the calculation of the figures for the total amounts of absorbed carbohydrate which could be accounted for on the basis of the data obtained, it was assumed that the total tissue glucose was equal to the

TABLE 4
Respiration in unfasted rats

	NUMBER OF RATS	O ₂ CONSUMP- TION	R.Q.	NON- PROTEIN R.Q.	CALORIES FROM CARBOHY- DRATE
		<i>cc. per 100 grams per hr.</i>			<i>per cent</i>
Normal rats	8	141	0.839 \pm 0.007	0.854	49
Hypophysectomized rats . . .	8	100	0.932 \pm 0.008	0.969	90

blood glucose distributed in 50 per cent of the body weight and that also the muscle glycogen was distributed in the same weight. The total liver glycogen was determined directly. As this table shows, in the normal uninjected rats, and in those injected for 20 days, about 85 per cent of the absorbed glucose was thus accounted for; but in the acute injection experiments, 70 per cent only was found. On the other hand, in the hypophysectomized rats, 96 per cent of the fed glucose seemed accounted for, but in the injected animals in this series, there was again a decrease in the total to 78 per cent. The probable errors of such an accounting, large as they must be, do not seem sufficient to explain these consistent deviations. Further work must be done to determine the significance of these differences.

From the data presented in the first three tables, it appeared that a substance was acting in the normal but not in the hypophysectomized rats which diminished the proportion of energy obtained from carbohydrate. It may have been that this substance was always present in the bodies of the normal animals; or that in these experiments its secretion had been

stimulated by the preliminary fasting period, for the prevention of otherwise rapid losses of body carbohydrate. For information to help decide this point, the respiration of *unfasted* normal and hypophysectomized rats was studied. The food intake of these animals prior to the experiments was checked in all cases; as shown previously (1), under these conditions the carbohydrate levels of hypophysectomized rats are quite normal. Table 4 gives the results obtained.

As far as can be judged from such figures, therefore, unfasted rats on a high carbohydrate diet also show the effects of hypophysectomy on the proportion of their energy which they obtain from carbohydrate.

DISCUSSION. The arguments presented here have been based on interpretation of the respiratory data in the usual manner, the emphasis having been placed on carbohydrate oxidation; but it might be considered that the changes observed in respiratory quotients in these experiments were produced by changes in gluconeogenesis. However, it seems unlikely that this process is concerned here, for in all cases there was a large excess of carbohydrate available—a situation in which gluconeogenesis should have been in abeyance. If the differences found between the normal and hypophysectomized rats were to be explained in this way, then there would have to have been a considerable amount of gluconeogenesis from fat in the normal rats fed glucose. Since there is now no evidence for such a conclusion, for the present the alternative interpretation has been used. Similarly, the excess of carbohydrate available makes it improbable that gluconeogenesis from protein could explain these results. Moreover, there were no significant changes in nitrogen excretion in these experiments.

The relationship of the phenomena described here to the action of insulin presents interesting problems. Since hypophysectomized animals are so sensitive to insulin, and anterior lobe extracts are capable of decreasing sensitivity to this substance, it may be thought that changes in oxidation of carbohydrate here observed are due to changes in the sensitivity of the animals to their own insulin. But it also seems likely that changes in insulin sensitivity may themselves be due primarily to the influence of the anterior lobe hormones on the oxidation of carbohydrate. It would be expected that the action of injected insulin would be greater in the absence of such a restraining influence on the oxidation of carbohydrate.

Differences between the carbohydrate metabolism of normal and of hypophysectomized animals have previously been observed chiefly during conditions of an "emergency" nature, such as fasting or phlorizin poisoning. From the data presented here, it appears that not only is the anterior pituitary secretion concerned with the preservation of body carbohydrate levels during interruptions in the supply of this substance, but it also plays a part in the disposition of fed carbohydrate. Its rôle again would appear to be one of suppressing the utilization of the carbohydrate.

SUMMARY

Hypophysectomized and normal rats, untreated and given injections of anterior pituitary extracts, were fasted and then fed known amounts of glucose. The oxygen consumption, respiratory quotients and N excretion were determined in the next several hours, after which determinations were made of the amounts of glucose absorbed and of the carbohydrate levels of the animals. Such determinations were also made on normal unfed rats with and without anterior lobe injections. The following results were obtained:

1. In the hypophysectomized rats, the proportion of absorbed glucose oxidized and the proportion of total calories obtained from carbohydrate were both much higher than in the normal rats. Decreases in the amount of glycogen stored were accounted for by the increases in the rate of oxidation of carbohydrate.

2. The injections of anterior lobe extracts into both hypophysectomized and normal rats caused an immediate fall in the apparent rate of oxidation of carbohydrate after this substance was fed. In the hypophysectomized rats, this level was brought below normal. More carbohydrate was stored as glycogen or as blood and tissue sugar by the injected rats.

3. Normal rats injected with the anterior lobe extract for 20 days became entirely resistant to the usual action of the extracts in suppressing carbohydrate oxidation and increasing its storage after feeding.

4. Normal unfed rats, in which carbohydrate oxidation was apparently already at a low level, showed no changes in R.Q. or carbohydrate levels when treated with the pituitary preparation.

5. Hypophysectomized rats which were unfasted (and which had ample carbohydrate supplies) had much higher respiratory quotients than did normal animals under the same conditions.

6. It was concluded that the anterior pituitary is concerned not only with the preservation of body carbohydrate during fasting, but also with the disposition of this substance when it is fed.

I wish to thank sincerely Dr. Herbert M. Evans for his interest and for his great material assistance in furthering this work. I must also thank Dr. Max Kleiber, Mr. L. L. Bennett and Mr. Charles W. Davidson for their efforts in designing and assembling the Kleiber respiration apparatus which I used.

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FACTORS INFLUENCING THE AUGMENTATION EFFECTS PRODUCED BY ZINC OR COPPER WHEN MIXED WITH GONADOTROPIC EXTRACTS

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Fevold, Hisaw, and Greep (1) have differentiated between the action of zinc and copper salts in augmenting the activity of gonadotropic extracts. They feel that "zinc salts probably produce their effect by decreasing the rate of adsorption of the active material. The activity of copper salts apparently cannot be explained on this basis but may be due to a catalytic action in the synergistic interaction of F.S.H. and L.H. in ovarian development." The augmentation effect of zinc reported by these workers was much less than that originally reported from this laboratory (2), while their results with copper compared favorably with those obtained by us for zinc (3). In the case of insulin we (4) had found that copper and zinc produced similar results. It seemed unlikely therefore that zinc should delay the resorption of both insulin and the gonadotropic extract, and that copper should produce the same effect upon one but not upon the other.

We were therefore led to study the conditions of precipitation of the pituitary gonadotropic extract by zinc and copper. This led naturally from the results obtained to the question of pH, the site of injection, and the influence of colloidal dispersion.

EXPERIMENTAL. The gonadotropic extract was the same used in earlier studies (5). Immature rats 22 to 23 days of age were used as the test animals. These were dosed once daily for four days and sacrificed the sixth day. Unless otherwise stated, the total dose per rat was 1.0 mgm. pituitary powder and 0.5 mgm. Zn or Cu as the sulfate.

Measures of variability. There is a considerable normal variation in the response to augmentation and unless the measure of variability is ascertained profound errors in deduction will arise. The following example illustrates this point. The normal ovarian weight of our rats on the day of sacrifice is 12 ± 2 (12 rats). Dosage of 1 mgm. gonadotropic extract produced ovarian weight of 17 ± 1 (18 rats). Two series of rats were dosed with the same preparation (1 mgm. per rat) to which was added 0.5 mgm. Cu as sulfate (pH 5.2). The ovarian weight of one series (6 rats) was 81 ± 12 ; the ovarian weight of the other series (12 rats) was

37 ± 5 . Each series demonstrates the profound augmentation produced by the addition of Cu, but the results of the two series differ between themselves by over three times the standard deviation of the mean. The variation in response can be reduced by comparison of litter-mates. With these the correlation coefficient is high, 0.85, and by comparison of litter-mates significance can be established when the treatment based upon a random population would leave one in doubt. Example: 32 ± 9 versus 69 ± 11 ; 2.6 times the standard deviation of the mean when treated as a random population, 4.2 times the standard deviation of the mean when a comparison of litter-mates is made.

Influence of pH of solution. Only data for litter-mates were compared in studying the effect of the pH of the solution injected in producing augmentation. In these cases the standard deviation should not be used as a criterion of significance. In the following experiments the solution was injected subcutaneously. Zinc produced a greater degree of augmentation when the solution injected was alkaline. Copper on the other hand produced the same degree of augmentation over the pH range 5.2 to 7.8. In the zinc experiments, the litter-mate which received the alkaline solution in every case had ovaries weighing more than those of the mate which received the acid solution.

Comparison of ovarian weights of litter-mates

Zinc: pH 5.0	26	33	23	87	16	20	19	32 average wt.
pH 8.5	42	107	72	101	76	37	49	69 average wt.

Comparison between pH 4.2 and 7.8 produced an even greater difference, 22 versus 68.

In the copper experiment, six litter-mates had larger, six litter-mates had smaller ovaries than their respective mates, the average ovarian weights being 37 versus 40. The pH was without effect.

A direct comparison of zinc and copper was made at pH 6.0 using litter-mates. At this pH copper produced the greater augmentation.

Comparison of ovarian weights of litter-mates

Zinc: pH 6.0	38	31	40	58	41	42 average wt.
Copper: pH 6.0	60	90	52	63	107	74 average wt.

At a neutral or alkaline pH little difference between the effect of zinc or copper could be ascertained. The results at pH 8.5 follow:

Comparison of ovarian weights of litter-mates

Zinc: pH 8.5	64	108	115	90	80	64	87 average wt.
Copper: pH 8.5	122	89	90	114	115	82	102 average wt.

Influence of site of injection. The experiments recorded in table 1 were performed upon litter-mates. The results are clear cut and demonstrate

that the site of injection plays an important rôle in the augmentation effect. Thus zinc shows little effect when the mixture is given intraperitoneally, less effect when given intramuscularly than when given intradermally. Copper is not as effective when given intradermally as when given subcutaneously.

Solubility of the Zn or Cu combination. Zn at pH 5.0. Twenty milligrams Zn as sulfate were added to a clear solution of 20 mgm. of pituitary powder in 10 cc. volume. The precipitate which formed (pH 5.0) was removed by centrifugation. The precipitate was taken back to 10 cc. volume and 10 mgm. Zn as sulfate added to both suspensions (filtrate and precipitate). Litter-mate rats were used for assay, a dose equivalent to 2 mgm. original powder per rat being used. The precipitate fraction produced ovaries weighing 25 mgm., the filtrate fraction produced ovaries

TABLE 1

Influence of site of injection on the effect of zinc and copper salt augmentation of gonadotropic extracts

	pH OF SOLUTION	INJECTION ROUTE	NUMBER OF TEST RATS	WEIGHT OF OVARIES
Zn added.....	5.0	Intradermal	7	34.5 \pm 4.5*
Zn added.....	5.0	Intraperitoneal	7	19.0 \pm 1.4*
Zn added.....	5.0	Intramuscular	7	24.0 \pm 2.5*
Control.....	6.0	Subcutaneous	6	17.0 \pm 1.8*
Cu added.....	5.2	Intradermal	6	46.0 \pm 5.5*
Cu added.....	5.2	Subcutaneous	6	81.0 \pm 12*

Dose: 1 mgm. pituitary powder plus 0.5 mgm. Zn or Cu as sulfate given in 4 doses on successive days.

* Litter-mates.

weighing 126 mgm. This experiment shows quite conclusively that the gonadotropic-activity factor is not precipitated by zinc salts at pH 5.0. (A slight amount of the factor is adsorbed.)

Zn at pH 8.5. The above experiment was repeated changing the pH to 8.5, before separating filtrate and precipitate. Doses equivalent to 1 mgm. original powder were used. The filtrate fraction produced ovaries weighing 18 mgm., the precipitate fraction produced ovaries weighing 51 mgm. This experiment shows quite conclusively that at pH 8.5 the active material is precipitated by zinc.

Cu at pH 5.0 and pH 8.5. The above experiments were repeated using copper sulfate instead of zinc. In addition to removal of the precipitates by centrifugation, the precipitates were washed once with water, which was then added to the filtrates. The pH 5.0 precipitate and the pH 8.5 filtrate produced ovaries (dose equivalent to 1 mgm. pituitary powder)

weighing 16 mgm. and showing slight if any effect. The pH 5.0 filtrate and the pH 8.5 precipitate produced ovaries weighing 32 and 53 mgm. respectively. The results therefore are like those found for zinc.

Injection of copper at a site removed. In four series of experiments comprising litter-mates, copper was injected at a site removed from the site of injection of the gonadotropic extract. In two experiments pituitary gonadotropic extract was used and in two other experiments prolan was used.

In a series of 7 pairs of litter-mates, the group receiving only gonadotropic extract (1 mgm.) showed ovaries weighing 17 ± 2.1 mgm.; the group receiving in addition copper (0.5 mgm.) injected subcutaneously at a site removed showed ovaries weighing 18 ± 1.9 mgm. In another series, a comparison was made between the subcutaneous and intraperitoneal injection of copper, in both cases at a site removed from the injection of the gonadotropic extract. Values of 20 ± 1 versus 22.5 ± 1.3 were obtained. In the prolan experiments male rats were used; they received 0.04 mgm. prolan and 0.5 mgm. Cu. Seminal vesicle weights of 23 ± 1.3 versus 26 ± 1.5 and prostate weights of 97 versus 98 were obtained. These experiments demonstrate quite conclusively that it is necessary for the copper to be mixed with the gonadotropic extract in order to produce augmentation. (Prolan effects are affected little by divided dosage, or dosage of suspensions in which $\text{Zn}(\text{OH})_2$ is the adsorbing agent. The prolan, copper experiments were tested to find out whether copper might function as suggested by Fevold, Hisaw, and Greep by some other mechanism.)

DISCUSSION. Most workers who have studied or discussed the augmentation phenomenon have failed to consider that augmentation may be produced simply by divided dosage (4) (5), and that this statement applies not only to the gonadotropic extracts but also to insulin, and the so-called growth-hormone extracts.¹ Since copper salts form insoluble precipitates with the gonadotropic preparation at the pH of the body and since in addition they produce considerable local necrosis when used in excess, it is unnecessary to assume that their action is any different from that of zinc or the now considerable list of other protein precipitants which produce the same effect. Their efficacy when used at a pH at which they do not form an insoluble compound and at which zinc has a lessened

¹ Unpublished data filed with the International Cancer Research Foundation. Squibb's Growth Hormone preparation in a single dose of 0.3 cc. per day brought about an 8 per cent gain (over controls) in body weight in 22 gram mice in 15 to 20 days. The same amount given in three doses per day brought about a 15 per cent gain (over controls) in body weight. When tested in groups of 8 to 15 animals, the difference between the control and single dose values, as well as the difference between the single dose and divided dose values was significant. Incidentally the test is an economical means of testing the activity of the growth hormone preparation.

effect is due doubtless to the precipitation with subsequent necrosis of tissue constituents at the site of injection, a process which entrains the active principle and delays its liberation most effectively. From a therapeutic standpoint copper is inferior to zinc not only from the standpoint of local necrosis but also because of the systemic toxic effect. As an agent to measure maximum gonadotropic activity, without resorting to the tedious process of divided dosage, it is superior even to zinc because the pH factor is eliminated.

The data on which Fevold, Hisaw, and Greep (1) base their deduction that F.S.H.² is augmented by zinc but not by copper in hypophysectomized animals and hence that the two metals exert their influence by different mechanisms bear inspection. If their control value, 13, for zinc, were substituted by their control value, 17.5, for copper, significance is barely established. Nor are their rabbit data convincing. Since ovulation in the rabbit is apparently brought about by nervous stimulation, it should not be surprising that the intravenous injection of 10 mgm. of as toxic a substance as copper acetate should lead to nervous stimulation and ovulation, while the relatively non-toxic³ zinc salt should produce no such result. In looking up our notes (prior to 1929) on studies of lead poisoning, we find that the intravenous injection of colloidal lead carbonate in a male rabbit produced a series of ejaculations over a half-hour period.

SUMMARY

1. The active pituitary gonadotropic fraction is not precipitated by zinc or copper salts at pH 5.0, but is precipitated at pH 8.5.
2. The maximum augmentation effect is produced when the pH of the injected solution is adjusted near the pH of precipitation.
3. Zinc salts are less effective than copper salts at an acid pH (pH 5.0) in producing augmentation, but are nearly as effective at a more alkaline pH (pH 8.5).
4. Copper or zinc salts injected separately and at a site removed from the gonadotropic extract injection site, produce no augmentation effect.
5. The site of injection of the augmentation mixture (intraperitoneal,

² Robson (J. Physiol. **90**: 125, 1937) has recently shown that the ovary of the rodent loses its capacity to gonadotropic stimulation as soon as three days after hypophysectomy. Moreover the luteinizing response is lost when growth of the follicular system can still be produced. Since F.S.H. fractions which consistently give negative luteal responses in normal immature animals have never been obtained, and since the test on the hypophysectomized animal would on the basis of Robson's experiments be open to serious objection, the isolation of a pure F.S.H. fraction is still open to doubt.

³ Unpublished data of G. J. Clark and F. Bischoff. In immature rats, 0.5 mgm. Cu produces thymus atrophy as measured by a 30 per cent decrease in thymus weight; 0.5 mgm. Zn produces no significant thymus weight change.

intramuscular, subcutaneous, intracutaneous) is an important factor in the degree of augmentation produced.

6. Copper salts produce more local necrosis than zinc salts.

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FURTHER STUDIES ON INTESTINAL ABSORPTION WITH THE PERFORMANCE OF OSMOTIC WORK

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Previous observations have shown the ability of the intestine to perform osmotic work in absorbing certain classes of substances. Following the earlier suggestive work of Katzenellenbogen (1), Goldschmidt and Dayton (2), and Burns and Visscher (3), we (4 and 5) showed that in the presence of polyvalent anions the univalent anions are moved from intestine to blood against as much as a 200-fold concentration gradient. In a preliminary note we (6) have reported that in the presence of a divalent cation the univalent cation sodium likewise is moved out of the intestine into the blood against steep concentration gradients. In this paper, we shall report the latter experiments in more detail, present additional studies on phenomena related to this function of the intestine, and finally outline an hypothesis which may in part account for the facts observed.

The methods employed in these studies are in general those described by us (4), except in specific cases which will be referred to in the description of the particular experiments.

I. *Uni-univalent salt impoverishment in the presence of a poly-univalent salt.* When an isotonic solution containing equi-osmotic fractions of NaCl and $MgCl_2$ is placed in a loop of lower ileum of the amytal anesthetized dog the course of the salt concentration change is as seen in figure 1. The final sodium concentration was 5 mM/l., as compared with 139 mM/l. in the blood into which it was absorbed. The ratio of 28 is not as great as the corresponding ratio for chloride of 200, observed in occasional instances (4) in the presence of polyvalent anions. Such a ratio could not be set up in arriving at any known membrane equilibrium (4).

In contrast to the changes in $[Na^+]$ it is seen that the $[Cl^-]$ arrived in 2 hours at approximately the blood plasma level for that ion. Thus the situation in this type of experiment is in essence the reverse of that found when a uni-polyvalent salt is present.

The $[Mg^{++}]$ is not entirely retained in the intestine during the period of the experiment. In fact one half or more of the total introduced may be absorbed from the intestine in 2 hours. In this particular experiment the

fluid volume was reduced from 50 cc. to 25 cc. Since the $[Mg^{++}]$ remained nearly constant it can be seen that a little more than 50 per cent was absorbed in this case.

Eight experiments of this type were performed and in each case the changes were the same in direction, and approximately the same in extent. In two experiments the bicarbonate content and pH were determined in addition to $[Na^+]$ and $[Cl^-]$, the former by the Van Slyke method and the latter with the quinhydrone electrode. The results for one experiment are given in table 1, where it can be seen that there is the characteristic impoverishment of the $[Na^+]$, an approach to the blood plasma level (100 mM) by the $[Cl^-]$, a large increase in $[HCO_3^-]$ and a significant shift to the alkaline side in the pH.

The change in pH is opposite to that observed when univalent anion impoverishment is brought about by the presence of polyvalent anions.

Other polyvalent cations have been used to attempt to obtain a comparable result. When an isotonic solution containing equiosmotic quan-

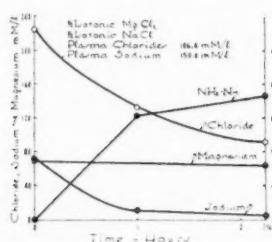


Fig. 1

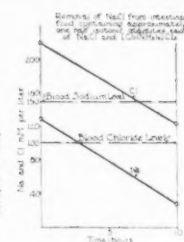


Fig. 2

ties of $AlCl_3$ and $NaCl$ is placed in the gut no specific absorption occurs. The concentrations of both Na and Cl approach their respective blood levels. The original pH is 3.37, however, which is an absolutely unphysiological acidity, and may account for the absence of a polyvalent ion effect by its toxicity. The end pH of the solution was 7.18.

$FeCl_3$ and $FeSO_4$ have actions similar to $AlCl_3$. With $CaCl_2$ there was some Na impoverishment. In 45 minutes the $[Na^+]$ fell to one-third of the blood plasma level. $MnSO_4$ likewise produced some Na impoverishment, its concentration falling to one-fourth of the plasma level in 45 minutes. The Cl impoverishment was somewhat greater in this case, its concentration falling to a fifth of the plasma level. The significance of simultaneous Na and Cl impoverishment will be discussed at greater length in the next section of the paper.

Two of the cobaltamine chlorides have been employed, the trivalent, and the hexavalent. The latter was ineffective, because of insolubility, we believe, but the trivalent cobaltamine $[Co(NH_3)_6]Cl_3$ had a marked

effect, causing a fall in $[\text{Na}^+]$ in the gut to one-fifth of the plasma level. Figure 2 shows the result of such an experiment. The action of this polyvalent cation seems particularly significant in demonstrating the non-specificity of the phenomenon.

To summarize, four polyvalent cations have been found to cause more or less impoverishment of the univalent cation in the gut, Mg^{++} , $[\text{Co}^{+++}(\text{NH}_3)_6]$, Mn^{++} , and Ca^{++} , named in decreasing order of effectiveness. The Fe^{+++} and Al^{+++} did not produce any impoverishment effect, but it is believed that their general toxicity at low pH and their low solubility at physiological H^+ concentrations will account for the absence of the effect. It seems legitimate to conclude that polyvalent cations when non-toxic at concentrations at or near isotonicity, produce removal of Na^+ from the intestine against large concentration gradients.

II. *Uni-univalent salt impoverishment in the presence of a poly-polyvalent salt.* Since it was observed that in the presence of a polyvalent cation Na

TABLE 1

Electrolyte changes in fluid in the ileal loop of the dog

One hundred cubic centimeters isotonic fluid ($\frac{1}{2}$ isotonic each with respect to NaCl and MgCl_2) introduced, 70 cc. recovered at the end of 2 hours. Amytal anesthesia.

TIME	Cl	Na	HCO_2	pH
	mM/l.	mM/l.	mM/l.	
0	211.9	70.2	0.0	7.03
2 hours	114.6	12.2	58.6	7.88

is absorbed against its concentration gradient into the blood and that in the presence of a polyvalent anion the univalent anions are so absorbed, it becomes of interest to note what might occur when a poly-polyvalent salt is introduced along with a uni-univalent one in the intestine. It might be predicted that under these conditions the univalent cation and anion would both tend to disappear from the fluid in the intestine. As will be seen, the results bear out this prediction. In figure 3 one sees the result of an experiment in which two adjacent loops of lower ileum were treated exactly alike, except that in one instance the solution was one-half isotonic with Na_2SO_4 and in the other instance with MgSO_4 . In the first case, as usual in such experiments, the $[\text{Cl}^-]$ is observed to fall to a small fraction of its blood level, in this instance to 9 mM. Within 45 minutes the $[\text{SO}_4^{--}]$ has increased while the $[\text{Na}^+]$ has declined only slightly, and at the end of 90 minutes was still 40 per cent above its blood plasma level. In contrast, the adjacent loop containing MgSO_4 showed an impoverishment with respect to the plasma level of both Na and Cl, along with an increase in concentration of the MgSO_4 . Comparable results were ob-

tained in each of the three experiments in which this procedure was followed. The $[\text{Na}^+]$ in each case diminished less rapidly than the $[\text{Cl}^-]$ but there was movement against large concentration gradients by both ions in every case.

It seems impossible to escape the conclusion that univalent ions can be transported out of the gut against large concentration gradients whenever a polyvalent ion of the same sign is present and only when that condition is satisfied. One reservation should perhaps be made to that generalization, namely, that when a non-ionized crystalloid which is absorbed slowly from the intestine is present a slight impoverishment has been observed. In a few experiments sucrose was used and the concentration of NaCl fell to one-third of its plasma level, but practically all of such solutions are absorbed in 45 minutes and therefore conditions are not optimal for the demonstration of the effect in question.

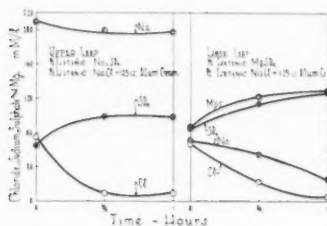


Fig. 3

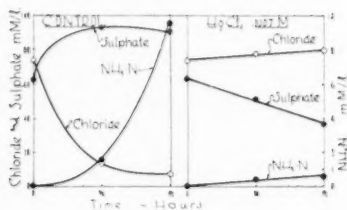


Fig. 4

III. *The importance of the pH in univalent ion impoverishment.* When isotonic mixtures of Na_2SO_4 and NaCl solutions are placed in the lower ileum there is regularly a decrease in pH of the fluid content over time, as Cl impoverishment is occurring. Fifteen experiments were performed and a typical example is presented in table 2. It will be noted that the pH of the originally unbuffered solution in the intestine fell from approximately 7 to 6.3, while the Cl in the intestine was falling to 4 per cent of the blood plasma level.

The $[\text{H}^+]$ and related factors seem to be of some importance to the speed and extent of the Cl movement. In four experiments a comparison was made of the Cl absorption from buffered and unbuffered solutions containing bicarbonate. In table 3 are shown the results of two consecutive absorptions from a single loop of gut. In the first instance 100 cc. of the usual NaCl and Na_2SO_4 mixture were introduced and in the second the same mixture containing 0.1 per cent NaHCO_3 . Several facts are to be noted in these results. It is seen first that there can be a Cl impoverishment of considerable extent without the gut-fluid being acid. In the second part of the experiment the pH did not fall below 7.19. It is ap-

parent, however, that the Cl movement was not as rapid in this case as in the control, which would indicate that the conditions were less favorable for the occurrence of Cl impoverishment. Furthermore, it will be seen that in the presence of NaHCO_3 the bicarbonate in the intestinal fluid may be nearly equal to that in the plasma without preventing the movement of Cl. The $[\text{HCO}_3^-]$ was calculated from the total CO_2 content and the pH using the Hasselbach equation and the value 6.1 for pK_1 . The H_2CO_3 content was determined by constructing a pH- CO_2 tension curve, and using the solubility factor $\alpha = 0.510$. The true value of the pK in the equation when applied to the buffer system in the intestinal fluid may not be 6.1. For the higher CO_2 values at high pH, however, the situation would not be materially altered by using any reasonable figure for this factor. It would

TABLE 2

The pH changes associated with Cl impoverishment in the ileal loop of the dog

	TIME	pH	Cl	TOTAL CO_2
	minutes		mM/l.	mM/l.
Gut fluid.....	0	7.05	73.3	0.0
	90	6.34	3.7	4.8
Plasma.....		7.18	101.7	24.5

TABLE 3

The effect of buffering with NaHCO_3 on the pH change and Cl impoverishment

GUT FLUID	TIME	pH	Cl	HCO_3
	minutes		mM/l.	mM/l.
Na_2SO_4 and NaCl.....	90	6.81	9.4	0.42
Na_2SO_4 , NaCl and NaHCO_3	90	7.19	26.7	16.25
Plasma.....		7.20	79.7	18.56

inevitably be true that Cl impoverishment could occur while a relatively high bicarbonate concentration was being maintained in the gut. It should be pointed out, however, that the figures obtained in this experiment were the highest we have ever obtained for gut fluid bicarbonate with the addition of NaHCO_3 to the intestinal fluid. Ordinarily we obtained between 2 and 6 mM/l. HCO_3^- under these circumstances.

In four additional pairs of experiments the pH of the intestinal fluid was altered by the addition of phosphate buffers and the effects of these differences noted. It was ordinarily found (3 times out of 4) that the absorption of Cl was faster from the acid buffered than from the alkaline buffered solutions. An example is given in table 4. In these experiments the phosphate was used as the polyvalent ion and the buffer mixtures were as indicated in the table. The remarkable ability of the intestine to alter

the pH of its contents is evident in this experiment in which it is seen that over the course of 90 minutes the pH in the two adjacent loops has approached the same figure, 6.1. The Cl absorption was significantly greater, however, from the more acid solution. In the one case in which there was no difference in the rate of absorption the Cl impoverishment was not satisfactory in either loop. As was noted in previous publications, the absorption against very high concentration gradients is not observed in every experiment. Chilling of the animal and excess of anesthetic are particularly unfavorable factors, and it may be that the failure of this experiment to conform in results to those of the others, is to be accounted for on the basis of the unusually poor absorption rate. The general regularity of the other results argues for this interpretation.

A few experiments have been performed in which the pH was measured when polyvalent cations were present in the intestinal fluid and univalent

TABLE 4
Effect of acid and basic buffers on chloride impoverishment

INITIAL CONTENT OF LOOP	TIME	pH	Cl	NH ₃ -N
	minutes		mM/l.	mM/l.
65 cc.				
0.065 M KH ₂ PO ₄	0	4.93	74.5	0.0
0.0017 M Na ₂ HPO ₄	45	6.20	24.2	0.9
0.074 M NaCl	90	6.06	5.6	1.4
65 cc.				
0.057 M Na ₂ HPO ₄	0	7.32	74.5	0.0
0.014 M KH ₂ PO ₄	45	6.84	30.0	0.7
0.074 M NaCl	90	6.12	16.3	1.7

cation impoverishment was occurring. Table 1 contains the results of the pH measurements in one such experiment. As was noted in the description of that experiment, the shift in pH is opposite to that observed when neutral salts of the uni-polyvalent type are introduced, and univalent anion impoverishment occurs. No observations have as yet been made on the influence of buffering upon the univalent cation impoverishment.

The effects of acid and alkaline buffers on Cl impoverishment lead one to conclude that the pH is at least a minor controlling factor in determining the rate of absorption in this case.

IV. *Ammonia accumulation and the univalent ion impoverishment.* Various authors have previously reported ammonia production by the intestine, and have given different interpretations to the findings. Folin and Denis (7) stated that the high content of ammonia in portal vein blood is due to bacterial action in the intestine. The literature on this subject before 1921 is exhaustively reviewed by Nash and Benedict (8). The methods

for ammonia determination in blood are so sensitive to differences in procedure that there is even today no agreement on the question of the true content of normal blood for this substance. The chief difficulty is, of course, in arranging conditions so that ammonia will not be freed from substances which might liberate it in an alkaline medium. We have not attempted to settle the arguments as to the ultimate validity of the ammonia methods but have followed the method of Nash and Benedict (8). The several samples were treated alike and therefore our results have comparative significance, regardless of whether the absolute values are correct, because we are comparing similar fluids at various times and after different experimental procedures. Within wide ranges the absolute values are not important for the purpose of our arguments, and we have reason to believe that within those limits the absolute values are correct. We are concerned chiefly with the contention that the true blood ammonia is normally very low, which is generally admitted, and the fact that relatively large quantities are formed in the intestine under the conditions of our experiments, which will, we believe, be evident from the data presented.

It was observed by Pendleton and West (9) that when isotonic NaCl solutions were placed in the bowel, urea entered the fluid, and that its concentration eventually exceeded the plasma level. They also noted that as much as 1 mgm. per cent of $\text{NH}_3\text{-N}$ accumulated in the fluid over a 2 hour period. This observation suggested to us the desirability of measuring the ammonia content of the intestinal fluid under the conditions of our experiments, and determining whether any correlation could be found between ammonia production or accumulation and univalent ion impoverishment. The ammonia content of the intestinal fluid and the blood was determined in experiments on 52 dogs. A number of questions were tested, the first being the regularity and magnitude of the increase in $\text{NH}_3\text{-N}$ in the gut over time. It should be noted that our methods (4) included the thorough flushing of the intestinal loop, until the washings were quite clear, and that samples of fluid were withdrawn and measured without contact with air. Blood was also handled without aeration, and $\text{NH}_3\text{-N}$ determinations made immediately.

Without exception ammonia accumulated in the fluid in the gut while univalent ion impoverishment was occurring. An example is seen in the left hand portion of figure 4, marked control. In this experiment an adjacent loop of gut was filled with the same solution plus 0.0007 M HgCl_2 . It will be seen that in this loop the results were entirely different. As we (5) have previously reported, Cl absorption against a concentration gradient is abolished by this poison, and in addition it is now seen that the ammonia accumulation is also virtually abolished. In figures 5 and 6 are presented the results of similar experiments, with their controls, in which NaF, and Na_2HAsO_3 in the concentrations noted, were used. With

these toxic substances, too, it is noted that the ammonia accumulation is inhibited when the Cl absorption is interfered with. Comparable experiments with NaCN and H_2S gave similar results. The simultaneous interference with both processes bespeaks an intimate relationship between active absorption and the metabolic processes yielding ammonia as one end product. It would be improper, however, to assign a direct causative relation between the high ammonia gradient from gut to blood and the anion impoverishment, first, because the poisons in question also make the intestinal epithelium more permeable to the polyvalent ions—(note the decrease in SO_4 concentration in these experiments as contrasted with their controls)—and second, because no invariable relation exists between high gut ammonia and rapid Cl movement. As noted before, in a small fraction of cases we do not obtain univalent ion impoverishment in the presence of polyvalent ions. Yet in these cases the gut ammonia rises as high as or higher than it does in the instances when rapid absorption occurs.

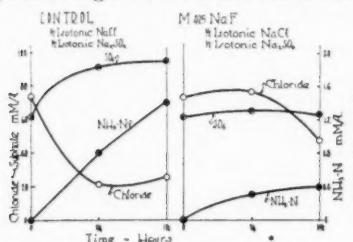


Fig. 5

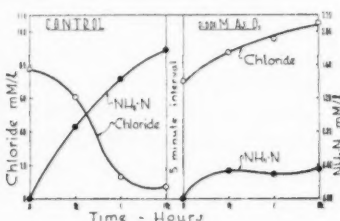


Fig. 6

(See table 5.) This might, of course, be due to changes in the membrane making a high ammonia gradient ineffective in influencing the processes in question, but we have no positive evidence bearing on this point.

In table 5 are presented figures for the simultaneous determination of ammonia in blood and in gut fluid at the time of maximum Cl absorption. In the 16 experiments for which these data are available 13 gave values less than 0.10 mgm. per cent for the blood NH_3-N . It happened that only those in which the blood NH_3-N was 0.10 mgm. per cent showed poor Cl absorption. It is very unlikely that this could be the result of an accident in sampling. We believe it to be very probable that a high blood ammonia is unfavorable to the occurrence of specific absorption and that a high $\frac{[NH_3]_g}{[NH_3]_b}$ ratio is favorable to such absorption. It may be that the absolute value of $[NH_3]_b$ is more important than the ratio, and that the blood ammonia elevation represents a specific metabolic disturbance which influences the absorbing activity of the intestine. There were no pH determinations made in this group of experiments, so it is impossible to calculate $[NH_4^+]$ with certainty, although when the pH is less than 7.0 a

negligible proportion of ammonia is undissociated. It is possible that changes in the pH of the blood would significantly alter the dissociation, however, because the blood pH range is critical for this dissociation.

To determine the influence of the blood concentration of ammonia more definitely, experiments were performed in which ammonium salts were injected intravenously while absorption was occurring from the intestine. In table 6 are presented the results of an experiment in which absorption was studied for two consecutive periods of 90 minutes each from the same loop of lower ileum. At the beginning of the absorption period in each case 50 cc. of a solution one-half isotonic each with respect to NaCl and

TABLE 5

Blood and ileal loop fluid $\text{NH}_3\text{-N}$ during Cl impoverishment in isotonic solutions originally containing equiosmotic fractions of NaCl and Na_2SO_4

EXPERIMENT NUMBER	$\text{NH}_3\text{-N}$		$[\text{NH}_3]_g$ $[\text{NH}_3]_b$	REMARKS
	Blood	Gut fluid		
	<i>mgm. per cent</i>	<i>mgm. per cent</i>		
14	0.02	3.33	167	Unless otherwise noted there was rapid Cl impoverishment
15	0.08	2.22	28	
16	0.06	2.50	42	
17	0.03	1.54	51	
18	0.06	5.46	91	
19	0.06	3.27	55	Poor Cl impoverishment Poor Cl impoverishment
20	0.04	4.14	104	
21	0.10	10.93	109	
22	0.10	2.66	27	
23	0.06	6.50	108	
25	0.04	10.66	262	No Cl impoverishment
26	0.10	4.49	45	
27	0.07	2.00	29	
28	0.03	2.50	83	
37	0.03	5.56	185	
42	0.05	2.96	59	

Na_2SO_4 were introduced into the loop. During the first period 4 cc. of a 5 per cent solution of NH_4Cl were injected into the femoral vein every 5 minutes. In this way 3.6 grams of the NH_4Cl were injected into a 10 kgm. dog. Although this quantity represents 36 mgm. per 100 grams of the dog's weight the maximum figure for $\text{NH}_3\text{-N}$ in the blood was 0.16 mgm. per cent. The dog with liver intact shows a remarkable capacity for removing ammonia from the blood. So long as the injection was at a rate compatible with life we have not been able to raise the blood $\text{NH}_3\text{-N}$ above ten times its pre-injection level, which ranged between 0.01 and 0.09 mgm. per cent. Six experiments of this type were performed and the results are in essential agreement. In spite of the repeated infusion of

NH_4Cl there was a very rapid Cl impoverishment in the intestine. From the point of view of establishing a relation between the ammonia gradient between the gut and the blood and the movement of Cl, however, these experiments are of no significance, because it will be noted that the level of ammonia in the gut was also greatly elevated by the intravenous injections. In every one of the six experiments there was an increase in blood ammonia brought about by the infusion.

The relatively enormous increase of gut fluid ammonia following intravenous injection of its salts presents an interesting problem in itself. Both during and after the injection the gut fluid $\text{NH}_3\text{--N}$ represents approximately 100 times its concentration in plasma. It could not, therefore, have reached this level by simple diffusion toward equilibrium. Most of

TABLE 6

Influence of intravenously injected NH_4Cl upon intestinal fluid $\text{NH}_3\text{--N}$ and other factors (Dog, amytal anesthesia, weight approximately 12 kgm.)

TIME AFTER INTRODUCING SOLUTION IN GUT	GUT FLUID			BLOOD		REMARKS
	Cl	Urea	NH ₂ -N	Urea	NH ₂ -N	
	mgm. per cent	mgm. per cent	mgm. per cent	mgm. per cent	mgm. per cent	
0	270.4	0.0	0.0	31.70	0.03	4 cc. 5 per cent NH ₄ Cl injected intravenously every 5 minutes for 90 minutes
45 min.	88.6	23.45	17.40			
90 min.	7.8	22.95	19.05	39.20	0.16	
Gut loop emptied and fresh solution introduced						
0	270.4	0.0	0.0			No injections
45 min.	23.3	33.26	3.53			
90 min.	5.8	37.19	4.14	31.26	0.04	

the ammonia injected into the blood is rapidly converted to urea, presumably by the liver. This is evidenced by the regular increase in blood urea which we observed in these experiments. There is also an increase in the urea in the intestine which in our experiments is approximately of the magnitude described by Pendleton and West. It might be supposed that urea were a precursor of the ammonia in the gut were it not for the fact that we have also in every experiment observed the gut urea to be highest during the second period of absorption when there is no longer increased urea production by the liver and the blood value for this substance is consequently falling, in this experiment from 39 to 31 mgm. per cent, and yet the ammonia in the gut is less than one-fourth of what it was at the lower gut urea level. We are led to suggest that there may be an intermediary between ammonia and urea circulating in the blood in appreciable quantities following injection of ammonium salts, which the intestinal epithelium may degrade to ammonia and which the liver is

able to carry to urea. We have not investigated the possibility that this may be citrulline.

The fact that intravenous injection of ammonium salts produces a very large increase in the $\text{NH}_3\text{-N}$ content of the gut provides, we believe, strong evidence that the ammonia in the intestinal fluid is of metabolic and not of bacterial origin. The introduction of various amino acids and urea directly into the gut in relatively large quantities, that is, 50 to 100 mgm. per cent, does not significantly increase the gut ammonia, which should happen, it would appear, if the ammonia were derived in the above circumstances from the bacterial metabolism of such precursors in the intestinal fluid.

The evidence at hand is insufficient to indicate exactly how the ammonia production is related to active absorption in the intestine. The simultaneous abolition of ammonia accumulation and active absorption of the type in question as a result of poisoning by many substances gives good ground for believing that the two processes are definitely interrelated. On the other hand, the fact that ammonia accumulation may occur without this type of active absorption proves at least that the presence of high ammonia is not the only factor necessary for the occurrence of the active absorption processes. One thinks of several ways in which the ammonia metabolism might be related to active absorption. It might be supposed that a high ammonia gradient between gut and blood might be responsible for the setting up of a membrane equilibrium of the type suggested by Teorell (10). Several facts preclude the possibility that such an interpretation is correct. First, the changes in concentrations are too great to be accounted for, and second, and more important, both anions and cations could not possibly be made to move simultaneously against concentration gradients on this basis.

One might suppose that the ammonia concentration differences would result in the setting up of a dynamic membrane potential resulting in abnormal osmosis as studied by Grollman and Sollner (11). This possibility leads to an attractive hypothesis as to the mechanism of water movement but the assumptions which must be made in order to employ it are not easy to justify experimentally. However, it should be pointed out that the processes of active absorption, like those of secretion, are themselves not simple and it seems too much to expect that an extremely simple system would be capable of bringing them about. The mechanism that we shall suggest in the last section of this paper is perhaps as simple as any which could be conceived to be capable of such selective processes.

A third possibility with regard to the relation of ammonia production to the process of active absorption should not be overlooked. It is not impossible that ammonia is a metabolic product of the chemical reactions going on in the intestine, the product itself having nothing directly to do with the dynamics of absorption.

Summarizing the results of the observations upon ammonia gradients

in this connection it may be said that active absorption has never been observed without a high gut to blood ammonia gradient. The ratios are as great as 250 to 1 and never less than 25 to 1. Various poisons which abolish absorption greatly diminish the ammonia accumulation. Occasional instances of high ammonia gradients without specific absorption have, however, been observed. It cannot be said, therefore, that a high ammonia gradient is the only factor necessary for univalent salt impoverishment.

V. *A consideration of a possible mechanism of the active absorption process.* Certain possible mechanisms by which specific active absorption might occur have been discussed previously (4).

As noted before, no ion exchange, or membrane equilibrium phenomenon has been found adequate to account for the various facts observed. Although the facts now available do not permit rigid proof of any theory it is now possible to outline a plausible hypothesis accounting for the known circumstances surrounding the process. This theory suggests that, in essence, the selective transport of materials against high concentration

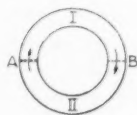


Fig. 7

gradients is a result of the circulation of fluid through differentially permeable membranes. If one conceives of the more or less continuous movement of water through the intestinal wall into the lumen of the gut through structures completely impermeable (or nearly so) to all electrolytes, and a simultaneous flow of fluid in the reverse direction from the gut to the blood through other structures permeable to uni-univalent salts but impermeable to polyvalent ion salts, the basic mechanism is available which will account for the phenomenon in question. We shall refer to this concept as the fluid circuit mechanism. Diagrammatically the system can be pictured as shown in figure 7, where A is a structure impermeable to all electrolytes and B is one impermeable only to polyvalent ion salts. If one postulates a driving mechanism for the continuous flow of fluid in the direction of the arrows, it is obvious that if I represents the intestinal fluid phase and II the blood phase, and if one starts with a mixture of uni-univalent and uni-polyvalent salts in compartment I, there will be a gradual decrease in concentration of uni-univalent salt in I, until eventually it will be present only in traces. This is the situation found by experiment in the living gut under these conditions.

The postulate of continuous active flow of fluid into and out of the gut is

not inconsistent with our general knowledge of intestinal epithelial structure. It consists of a mosaic pattern of various structures, even microscopically, and in dealing with such problems as fluid flow one might easily conceive of a mosaic pattern of sub-microscopic dimensions. Without committing oneself to such a simple theory one can point to the obvious facts that the intestinal glands are sites of flow of fluid from blood to gut, and that the villi are structures in which flow of fluid is toward the blood. The objection might be raised that the secretion of the intestinal glands, as it has been collected is not free from electrolyte, but contains significant quantities of NaHCO_3 . A small amount of this salt in the fluid from this source fits the facts observed in section II of this paper, in which it was noted that univalent cation impoverishment is always less complete than anion impoverishment. Moreover it should be pointed out that the composition of fluid collected from intestinal loops is not a satisfactory measure of the character of the secretion itself, since such fluid immediately flows over absorbing structures and is thereby altered in chemical composition. This fluid represents the algebraic sum of secretion plus absorption processes. It might be, and very probably is, considerably concentrated with respect to certain constituents by virtue of water absorption, and it may furthermore have materials added to it by diffusion through the epithelium.

Peters and we (12) have developed a mathematical expression describing the process pictured in figure 7, assuming constant rates of fluid flow at A and B, and with the other assumptions mentioned in the discussion of the theory above, and it is found that the expression proposed fits the experimental facts satisfactorily, within the limits of error of measurement. The integral of the differential equation describing the physical process in figure 7 is:

$$C = C_0 \left(\frac{V}{V_0} \right)^{\frac{R_i}{V_0 - V/t}}$$

where C is the concentration of uni-univalent salt at any time t , C_0 is the concentration at zero time, V_0 is the original volume, V the volume at time, t , and R_i is the rate of movement of fluid into chamber I (the intestinal lumen). For a complete derivation of this expression the reference above (12) should be consulted. In this expression R_i alone is incapable of direct measurement. It can be calculated by determining the other quantities at any two times. One notes that the value for R_i is substantially constant over 90 minutes of absorption. It is of some importance to observe that this equation is based upon the conditions of a real physical situation and that although R_i is mathematically an arbitrary constant it has a definite postulated physical significance.

If the fluid circuit mechanism is to be a plausible concept the calculated values for R_i must be of reasonable magnitude. In several experiments this value has ranged around 30 cc. per hour for a 20 cm. loop of lower ileum. This approximates 0.5 cc. per square centimeter of epithelial surface per hour. It is obviously not unreasonable to suppose that such a rate of flow might exist. If only a small proportion of the total surface were involved in the process, which is likely, there would still be need of only relatively slow water movement in order to account for the phenomenon.

The observed and calculated data for a typical experiment are presented in table 7. The observations extended over 1.5 hours and by comparison of columns 2 and 6 it will be seen that until the chloride concentration fell to 2 mM there is satisfactory agreement between observed and theoretical

TABLE 7
Observed and calculated data on the rate of active absorption

$$V_0 = 75 \text{ cc.} \quad V_{1.5 \text{ hrs.}} = 36 \text{ cc.}$$

$$\frac{V_0 - V}{t} = \frac{39}{1.5} = 26 \text{ cc./hour}$$

TIME	C EXPERIMENTAL	C C ₀	V	V V ₀	C THEORETICAL
<i>hours</i>	<i>mM/l.</i>		<i>cc.</i>		
0	59.5	1.00	75.0	1.00	59.5
0.167	40.1	0.67	70.7	0.94	38.7
0.333	28.7	0.48	66.3	0.88	24.5
0.5	15.6	0.26	62.0	0.83	16.3
1.00	3.5	0.059	49.0	0.65	3.0
1.5	2.2	0.037	36.0	0.48	0.36

figures. At this low level analytical methods are inaccurate and second order errors in the assumptions underlying the theoretical equation may enter in, particularly the assumption of complete impermeability of membrane A to all electrolytes might be in error. If it were permeable to only 2 per cent of the electrolyte in the fluid passing through it the deviation observed would be accounted for.

The nature of the force driving the fluid through the membranes is a separate problem. It is the usual view that electrical forces operative at the membranes may account for fluid flow by abnormal osmosis. The work of Söllner and Grollmann (11) elucidates the kinetics of these processes, but it is not possible with the facts now available to demonstrate that all of the conditions necessary for such movement are satisfied in the intestine. Further studies in this field are indicated.

In summary, it is believed that the fluid circuit mechanism postulated will account for many of the known facts of the secretory or active absorption process under consideration. The driving mechanism for fluid has not

been demonstrated, but active movement of water is well known to occur. It is therefore not unreasonable to postulate its occurrence in such a theory.

CONCLUSIONS

1. In the presence of poly-univalent salts uni-univalent salts are absorbed from the intestine until the concentration of the univalent cation is as much as twenty-eight times as great in the blood plasma as in the intestinal fluid from which it is moved. Osmotic work is performed on the univalent cation under such circumstances.

2. When a poly-polyvalent salt is introduced along with a uni-univalent salt the latter is absorbed against concentration gradients as great as ten to one. This behavior would have been predicted from previous observations on the polyvalent ions of each sign separately.

3. While univalent anion impoverishment is occurring in the intestine the pH of the gut fluid invariably becomes more acid. Experiments with the addition of bicarbonate and phosphate buffers show, first, that anion impoverishment will occur in spite of maintained alkalinity, and, second, that the gut tends to bring the pH of the contents to 6.1 to 6.3 regardless of the original pH value, even with strongly buffered solutions. In general univalent anion impoverishment is more rapid from the more acid solutions. During univalent cation impoverishment the gut fluid becomes more alkaline reaching pH 7.9.

4. Ammonia production occurs in the gut and the concentration of $\text{NH}_3\text{—N}$ in the fluid increases to as much as two hundred and fifty times its concentration in the blood during active absorption involving osmotic work. Poisons which result in the abolition of active absorption all inhibit the accumulation of ammonia. The possible significance of ammonia production is discussed.

5. A fluid circuit mechanism is suggested in an attempt to account qualitatively and quantitatively for the phenomenon of active absorption by the intestine.

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EFFECTS OF ANDROGENIC STEROLS IN HYPOPHYSECTOMIZED AND IN CASTRATED RATS

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In rats injected with suitable androgenic substances shortly following hypophysectomy the following results have been observed: maintenance of the testicular tubules (1, 2, 3, 4); atrophy of the interstitial cells (2, 3, 4); maintenance of the accessory sexual organs, including the scrotum (3, 4). If, however, the injection of androgens into hypophysectomized rats is begun after involution of the seminiferous epithelium has resulted, reestablishment of spermatogenesis does not occur (2, 3), but repair of the accessory glands (2, 3) does occur. We have suggested the possibility that the maintenance of the seminiferous tubules in hypophysectomized rats is due to an indirect action resulting from scrotal maintenance caused by male hormone (3, 5). Nelson and Merkel (4), however, feel that scrotal maintenance plays a relatively minor rôle in the maintenance of spermatogenesis in hypophysectomized rats treated with androgens, because the capacity of these substances to maintain the testis tubules was unrelated to their androgenic ability or to their ability to maintain the scrotum. In this connection it might be pointed out that lack of correlation between a substance's ability to maintain testis tubules in hypophysectomized rats and its androgenic activity does not necessarily rule out the possibility that tubular maintenance in hypophysectomized rats may be caused indirectly through scrotal maintenance. As a matter of fact it has been demonstrated (6) that yeast, non-androgenic when judged by the atrophic condition of the seminal vesicles and prostate, is capable of maintaining the testis tubules in hypophysectomized rats. It seems of significance that yeast was also observed to cause scrotal maintenance. With regard to scrotal maintenance, it may be questioned whether variations in the gross appearance of the scrotum necessarily indicate different degrees of scrotal function.

In a previous article (3) we reported the effects produced in hypophysectomized male rats treated with androsterone, testosterone, and dehydroandrosterone benzoate. Since then we have made similar studies with five other synthetic steroids:¹ Δ^5 dehydroandrosterone acetate, Δ^5

¹ Supplied through the courtesy of Dr. Erwin Schwenk, Schering Corp., Bloomfield, New Jersey.

TABLE 1

Average body and organ weights of rats injected subcutaneously for 18 days with various androgenic substances

	NUMBER OF RATS	INITIAL BODY WEIGHT	AUTOPSY WEIGHTS			
			Body	Testes	Seminal vesicles	Ventral prostate
Group I. Dehydroandrosterone acetate, 1.5 mgm. daily						
		grams	grams	grams	grams	grams
Hypophysectomized injected.....	4	288	249	2.17	0.524	0.172
Hypophysectomized control.....	2	286	225	0.81	0.150	0.054
Normal control.....	4	279	287	2.57	1.071	0.384
Castrated injected.....	4	285	303		0.147	0.116
Castrated control.....	3	266	278		0.145	0.049
Group II. Dehydroandrosterone acetate, 1.5 mgm. daily						
Hypophysectomized-castrate in- jected.....	5	195	161		0.082	0.056
Hypophysectomized injected.....	3	200	171	2.04	0.408	0.133
Normal control.....	3	189	218	2.45	0.734	0.202
Castrated injected.....	4	201	222		0.099	0.074
Castrated control.....	2	192	226		0.099	0.023
Group *III. Androstenediol, 1.5 mgm. daily						
Hypophysectomized injected.....	5	162	142	1.93	0.655	0.136
Hypophysectomized control.....	2	170	139	0.41	0.034	0.011
Normal control.....	8	129	162	2.40	0.424	0.146
Castrated injected.....	4	152	174		0.151	0.098
Castrated control.....	3	150	179		0.042	0.015
Group *IV. Androstenediol, 3.0 mgm. daily						
Hypophysectomized injected.....	5	157	121	1.85	1.009	0.214
Castrated injected.....	4	151	173		0.872	0.235
Group *V. Testosterone propionate, 1.5 mgm. daily						
Hypophysectomized injected.....	5	156	137	1.79	1.758	0.364
Castrated injected.....	4	149	170		1.380	0.485
Group **VI. Testosterone oxime, 1.5 mgm. daily						
Hypophysectomized injected.....	7	172	153	1.03	0.243	0.102
Normal control.....	5	159	193	2.45	0.629	0.221
Castrated injected.....	4	158	205		0.174	0.101
Castrated control.....	2	158	188		0.051	0.020

* and ** indicate groups having the same controls.

TABLE 1—*Concluded*

	NUMBER OF RATS	INITIAL BODY WEIGHT	AUTOPSY WEIGHTS			
			Body	Testes	Seminal vesicles	Ventral prostate
Group **VII. Testosterone oxime, 0.75 mgm. twice daily						
Hypophysectomized injected.....	5	grams 170	grams 147	grams 0.99	grams 0.218	grams 0.090
Castrated injected.....	4	146	182		0.104	0.094
Group VIII. Testosterone oxime propionate, 1.5 mgm. daily						
Hypophysectomized injected.....	4	146	136	1.00	0.516	0.124
Hypophysectomized control	2	145	133	0.55	0.040	0.011
Normal control	6	135	182	2.36	0.527	0.194
Castrated injected	4	157	212		0.246	0.125
Castrated control	2	155	210		0.058	0.017

androstenediol, testosterone oxime, testosterone oxime propionate, and testosterone propionate. The dose levels at which these substances were injected, together with other pertinent data, will be found in table 1. Injections in the groups given in table 1 were uniformly begun one day after operation and continued daily for 17 additional days; the animals were sacrificed on the day following the last injection. All organs were weighed in the fresh state and then fixed. Pituitaries and pituitary fossae were fixed in Helly's fluid, the sexual organs in Bouin's fluid. Helly-fixed tissues were stained with Mallory's triple stain and the sexual glands were stained with Delafield's hematoxylin and eosin. The quantitative results presented in table 1 are expressed on a percentage basis in table 2. For the sake of comparison the results we obtained with the three androgens previously investigated (3) also have been included in table 2. Table 3 shows the results obtained with injections of testosterone propionate or androstenediol in rats which had been hypophysectomized for more than 6 weeks before injections were begun.

DISCUSSION OF RESULTS. All the data in table 1 are not readily amenable to direct comparison, as all the experiments were not conducted concurrently and as there was considerable variation in body weight among some of the groups. All animals within a group, however, and in some instances those in different groups (e.g., III, IV, V), were of uniform stock. Usually several litters were divided so that each group contained experimental and control animals from the same litters. While absolute weight values in any one group may therefore be compared directly, values in different groups would seem to be more accurately compared on a relative basis. Table 2 represents relative weight values with normal control weights for their respective groups considered as 100.

TABLE 2

*Androgenic substances listed in descending order of potency in maintaining organ weights in hypophysectomized and in castrated rats injected daily for 18 days with 1.5 mgm. of substance**

TESTES	SEMINAL VESICLES		VENTRAL PROSTATE	
Dehydroandrosterone acetate (84.4)	Testosterone propionate (414) (326)		Testosterone propionate (249) (332)	
Androstenediol (80.4)	Androstenediol (3 mgm.) (238) (206)		Androstenediol (3 mgm.) (147) (160)	
Androstenediol (3 mgm.) (77.1)	Androstenediol (155) (36)		Androstenediol (93) (67)	
Testosterone propionate (74.6)	Testosterone (136)		Testosterone (76)	
Androsterone† (65)	Testosterone oxime propionate (98) (47)		Androsterone (74)	
Testosterone† (59)	Androsterone (58)		Testosterone oxime propionate (64) (64)	
Testosterone oxime propionate (42.4)	Dehydroandrosterone acetate (49) (14)		Testosterone oxime (46) (46)	
Testosterone oxime (42.0)	Testosterone oxime (39) (28)		Dihydroandrosterone benzoate (1.25 mgm.) (46)	
Dihydroandrosterone benzoate (1.25 mgm.)† (20)	Dihydroandrosterone benzoate (1.25 mgm.) (38)		Dehydroandrosterone acetate (45) (30)	
Hypophysectomized control (Maximum) (Minimum) (31.5) (17.1)	Untreated control (Maximum) (Minimum) (14) (11)		Untreated control (Maximum) (Minimum) (10) (13)	

* The relative degrees of maintenance (normal control values considered 100) are given in parentheses. Under the seminal vesicles and prostate the maintenance percentage for hypophysectomized rats is first and the corresponding figure for castrated rats is second.

† Cutuly, E., D. R. McCullagh and E. C. Cutuly. This Journal **119**: 121, 1937.

Testes. Despite the different degrees of testicular weight maintenance produced by dehydroandrosterone acetate, androstenediol (1.5 mgm.), androstenediol (3 mgm.) and testosterone propionate (table 2), the testes of the animals in these different groups were histologically similar. In each group there were some testes the tubules of which seemed entirely normal, some in which isolated tubules showed varying degrees of atrophy, and still other testes in which there could be seen numerous areas showing profound tubular degeneration. It was unexpectedly found that on the average there was no marked histological difference among the testes maintained by dehydroandrosterone and those maintained by testosterone propionate, although there was a relative weight difference of 10 per cent between these groups. The weight differences in these two groups cannot

TABLE 3

RAT NUM- BER	TOTAL NUMBER OF DAYS HYPO- PHYSEC- TOMIZED	INJECTIONS BEGUN DAYS AFTER HYPO- PHYSEC- TOMY	TREATMENT	NUMBER OF IN- JECTIONS	AGE AT START	AUTOPSY WEIGHTS		
						Testes	Semi- nal vesicles	Ventral Pros- tate
					days	grams	grams	grams
1	92	63	Testosterone propionate (3 mgm. daily)	28	50	0.37	1.458	0.252
2	93	64		28	49	0.28	0.707	0.229
3	73	47	Androstenediol (3 mgm. daily)	26	66	0.53	1.077	0.151
4	73	47		26	66	0.48	1.284	0.184
5	73		Hypophysectomized control		92	0.34	0.042	0.012
6-8			Normal control (average 3 rats)		49-66	2.59	0.965	0.377

be regarded as especially significant at this time, as there was a great inequality between the original body weights. To what extent age, initial body and testis size and dosage might have influenced the response in these studies is not definitely known. These are all factors which cannot be ignored in an analysis of the comparative abilities of androgens to maintain the tubules in hypophysectomized rats. We already have, in fact, unpublished data which seem to support this view. As might be expected from the weight values, there was extensive atrophy seen in the testis tubules of the hypophysectomized rats injected with the oxime in single and divided dose and the propionate oxime of testosterone.

In every case the interstitial cells of hypophysectomized rats injected with androgenic substances showed atrophy comparable with that observed in control hypophysectomized animals.

Scrotum. It is quite possible to distinguish an atrophic scrotum from

one which is normal, but it is another matter to distinguish intermediate grades of maintenance, if such grades actually exist. Our descriptions of scrotal maintenance are based on the intensity of flushing and the extent of swelling observed in the scrotal sac. No attempt is made to correlate intimately the gross appearance of the scrotum with its functional capacity. It would be of interest to know whether such a correlation might exist. Of the substances used in this study only testosterone oxime, in single or divided doses, failed to give at least a moderate degree of scrotal maintenance in the hypophysectomized rats. In the castrated injected rats, on the other hand, androstenediol at both dose levels employed, as well as testosterone oxime, produced no perceptible scrotal stimulation. Testosterone oxime propionate and dehydroandrosterone seemed to induce a moderate amount of scrotal maintenance in castrated rats, but testosterone propionate caused the scrota of castrated rats to appear not only extremely flushed but markedly turgescient.

Accessory sexual organs. With one exception all the substances employed caused histological maintenance of the prostate and seminal vesicles in hypophysectomized and in castrated rats. Dehydroandrosterone acetate failed to maintain the seminal vesicles in castrated rats, although it did prevent epithelial atrophy of the seminal vesicles in hypophysectomized rats; in hypophysectomized animals treated with this androgen the prostate was histologically stimulated, but in similarly treated castrates the prostate was undergoing atrophy. The other androgens all induced marked stimulation of the epithelium of the seminal vesicles and prostate in both hypophysectomized and castrated rats. It might be said that the great differences in weight of the accessories among the groups probably were largely due to the variable amounts of secretion present.

An interesting observation is that in some instances in the same group the relative weight of organs in hypophysectomized rats was greater than the corresponding value for castrated rats. That the presence of the testes was somehow responsible for this divergence was illustrated by the results obtained in hypophysectomized-castrated rats injected with dehydroandrosterone acetate (table 1, group II). Here it is seen that a dose of dehydroandrosterone acetate which produced a definite stimulation in the seminal vesicles and prostate in hypophysectomized rats, caused no stimulation whatsoever of the seminal vesicles and a very questionable stimulation of the prostate in castrated or in hypophysectomized-castrated rats. In the absence of the testes the effectiveness of this androgen was obviously reduced. Whether the testes in hypophysectomized rats contained or secreted a residual amount of testicular hormone which augmented the action of the injected substances, or whether they converted the injected material to a more active form are questions which cannot yet be answered.

Pituitary. Under the conditions of these experiments dehydroandrosterone acetate, androstenediol at the 1.5 mgm. level, and testosterone oxime had no apparent effect on the anterior lobes of the hypophyses of castrated rats. Testosterone oxime propionate, androstenediol at the 3 mgm. level, and testosterone propionate, however, prevented to varying extents the increase in basophiles which ordinarily follows castration and in some instances had a tendency to induce degranulation of the basophiles. No apparent effects were observed on the eosinophilic cells in any of these glands.

Postponed injections. From the results in table 3 it will be seen that testosterone propionate and androstenediol each given daily at a 3 mgm. level to hypophysectomized rats failed to have any significant stimulating effect on the tubules of testes which had undergone involution. While the seminal vesicles and prostates of these injected animals were well stimulated, the scrota did not show comparable stimulation at the end of the injection period. Both androgens seemed to cause marked growth and vascularization of the scrota of the hypophysectomized rats during the first third of the injection period. This scrotal response became less and less marked until it seemed, toward the end of the injection period, that regression of the scrotal sac would occur in spite of continued injections. During the period when the scrota of the injected hypophysectomized rats showed definite stimulation, the degenerated testes descended into the scrota, but subsequently, as the scrota regressed, the testes seemed to ascend. The reason for this inability of androstenediol and testosterone propionate to cause sustained scrotal stimulation is unknown. These observations indicate the need for further studies on the effects of androgenic substances in hypophysectomized rats.

CONCLUSIONS AND SUMMARY

Hypophysectomized and castrated male rats were injected daily for 18 days with 1.5 mgm. each of dehydroandrosterone acetate, androstenediol, testosterone propionate, testosterone oxime, and testosterone oxime propionate. Androstenediol was also injected at a 3 mgm. level.

In the testes of hypophysectomized rats injected with these substances varying degrees of tubular maintenance were induced, but in no instance did such treatment prevent atrophy of the interstitial cells. It was pointed out that a greater weight of maintained testes did not necessarily predicate a superior state of tubular preservation. The necessity of considering such variables as initial body and testis weight was indicated.

In hypophysectomized rats the testis tubules of which were maintained by androgens, varying degrees of scrotal flushing and turgescence were noted. It is not known whether such gross changes can be intimately correlated with scrotal function.

The seminal vesicles and prostate in hypophysectomized and in castrated rats were maintained to different extents by the androgenic sterols employed. Evidence was presented that the testes in hypophysectomized rats in some way augmented the androgenic effect of these sterols.

Some of the androgenic substances prevented increase in basophiles in the anterior hypophyses of castrated rats and in some instances also caused basophilic degranulation; no effect was observed on the eosinophiles.

Androstenediol and testosterone propionate injected at 3 mgm. levels failed to produce in hypophysectomized rats any significant gross or histological stimulation of testes which had undergone involution before injections were begun; these substances also seemed incapable of causing sustained serotal stimulation, although they did bring about repair of the prostate and seminal vesicles.

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THE EFFECTS OF VARIOUS GONADOTROPIC SUBSTANCES
UPON THE OVARIES, PITUITARIES AND ADRENALS OF
ANIMALS RECEIVING LONG-TERM INJECTIONS OF ESTRIN¹

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Within recent years abundant evidence that estrin exerts an influence upon the activity of the pituitary, ovaries and adrenals has been obtained; much work has been done in an effort to determine the exact nature of this influence. A number of investigators have reported that the injection of estrin for a comparatively short period of time produces hypertrophy of the pituitary (1-5), ovaries (1-5) and adrenals (3-5). These changes have been attributed to a stimulative action of estrin upon the pituitary, resulting in an increased secretion of gonadotropic and adrenotropic substances (3-5). The administration of estrin over a long period of time, on the other hand, has been observed to cause ovarian atrophy (6-7). Variable effects of such treatment on the adrenals have been reported (6-7). The pituitaries of long-term estrin-injected animals have been shown to exhibit a diminished ovarian stimulating capacity when implanted into immature animals (6, 8).

The changes resulting from long-term estrin administration have been interpreted in several ways. It has been suggested that such treatment causes inhibition (6, 9) or depression (10, 11) of hormone secretion by the pituitary or an overstimulation of the pituitary, leading to a state of exhaustion (12). These and other theories that have been advanced (6, 13) have been based chiefly on observations of changes which take place in the pituitary; little consideration has been given to the other glands affected indirectly by the injections. No attempt has been made heretofore to determine the nature of the ovarian atrophy which takes place. If this atrophy is due to a decrease in the amount of gonadotropic hormone available to the ovary, this is strong evidence that long-term estrin administration leads to a diminished secretion of the pituitary hormones. If, however, these atrophic ovaries fail to respond to added pituitary hormone, other hitherto unrecognized possibilities must be taken into consideration. It was our opinion, therefore, that a study of

¹ This work has been aided by a grant to Vanderbilt University School of Medicine from the Division of Medical Sciences of the Rockefeller Foundation.

the physiologic state of ovaries which had atrophied as a result of long continued estrin administration would furnish additional evidence as to the mode of action of estrin upon the pituitary and related glands. Accordingly, an investigation was made of the action of gonadotropic hormones and of massive doses of estrin upon such ovaries.

EXPERIMENTAL METHODS. A large number of mature female rats were injected with 200 rat units of estradiol benzoate (Progynon B)² daily for

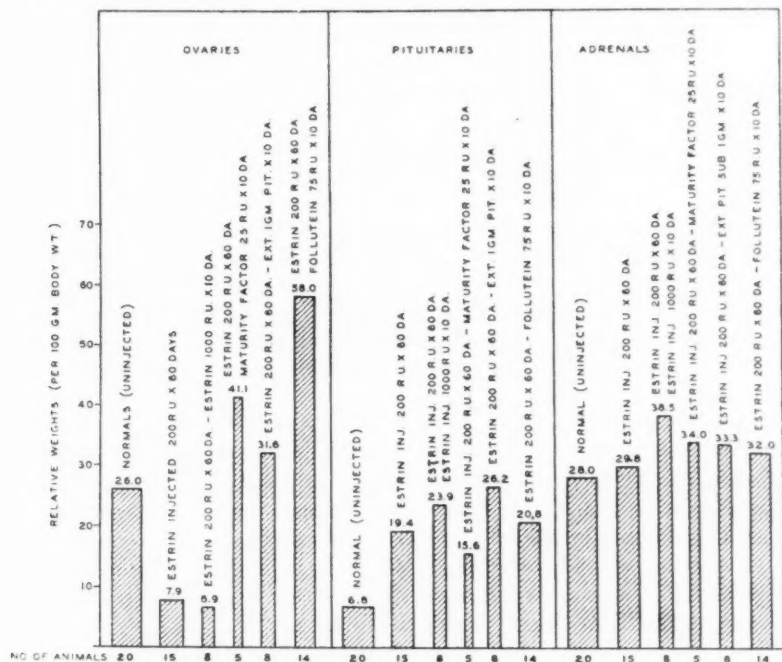


Chart 1. Graphic representation of average weights of ovaries, pituitaries and adrenals of animals receiving long-term injections of estrin and long-term injections of estrin followed by gonadotropic hormones and massive doses of estrin.

60 to 70 days. Beginning on the 60th day of the experiment, 35 of these animals received additional treatment as follows:

a. Twenty-seven animals were given, in addition to estrin, daily injections of gonadotropic hormone. The gonadotropic substances used included anterior-pituitary-like hormone (Follutein),³ commercial extract

² Supplied gratis by the Schering Corporation through the courtesy of Dr. Erwin Schwenk.

³ Supplied gratis by E. R. Squibb & Sons through the courtesy of Dr. J. A. Morrell.

of whole anterior pituitary (maturity factors) and a saline suspension of fresh hog anterior pituitaries. These substances were given in substantial doses for 5 to 10 days (chart 1).

b. Eight animals were injected for 10 days with 1,000 rat units of estrin (Progynon-B) daily.

Fifteen of the 60-day estrin-injected animals served as estrin-injected controls, and 20 normal mature female rats were used as uninjected controls.

All of these animals were sacrificed at the end of the injection period and the ovaries, pituitaries and adrenals weighed on a torsion balance (Hartmann-Braun). Serial sections of the ovaries and representative sections of the uteri and vaginae were studied histologically. Follicle counts were made on five representative ovaries from each group of animals. For this purpose, five equidistant sections of each ovary were selected. The follicles present in these sections were counted and classified according to the degree of development as follows: *primordial follicle*, an ovum surrounded by a single layer of simple flat epithelium; *primary follicle*, a larger ovum surrounded by a single layer of cuboidal epithelium; *growing (secondary) follicle*, an ovum surrounded by two or more layers of granulosa cells and a definite theca folliculi; *vesicular follicle*, one showing a definite antrum. The corpora lutea were also counted and included in the total follicle count.

The pituitaries of a small group of 60-day estrin-injected animals (8) and of a somewhat larger group of estrin and gonadotropic hormone injected animals were implanted, singly or in pairs, into immature mice. The ovaries of the mice so treated were compared with the ovaries of a number of normal immature mice used as controls.

Five 60-day estrin-injected animals were kept in a cage with a male for a period of four months following the cessation of treatment. Vaginal smears were obtained daily during this interval.

EXPERIMENTAL RESULTS. The ovaries of the 60-day estrin-injected animals were markedly decreased in size and weight, as compared with the series of normal rat ovaries (chart 1). There were no corpora lutea in these ovaries and the vesicular follicles were reduced in number (table 1). Most of the follicles of all types (except primordial) showed unmistakable signs of atresia. The interstitial tissue was decreased in bulk and the individual cells were small. No mitoses were observed.

The ovaries of the animals which received, in addition to estrin, 5 to 10 daily injections of gonadotropic hormone were greatly increased in weight; indeed, the average weights of these ovaries exceeded the average weight of the normal ovaries which served as controls (chart 1). The histologic findings varied somewhat with the gonadotropic substance injected, but in each case there was unmistakable evidence that stimula-

tion of the follicular apparatus of the ovary had been effected. Corpora lutea were present in every instance and there was practically no follicular atresia. There was, in addition, a marked increase in the amount of interstitial tissue with hypertrophy of the individual cellular elements. The theca interna cells were hypertrophied and increased in number, forming a thick layer around many of the follicles. No qualitative variations in the ovarian response referable to the particular gonadotropic substance administered could be detected. It was our impression that both follicle growth and luteinization were stimulated, regardless of the gonadotropic preparation used, and that the corpora lutea present in each case were essentially normal in that they were composed chiefly of granulosa elements. Such variations as were observed in the different groups of ovaries appeared to be entirely of a quantitative nature, dependent upon the potency of the substance injected.

TABLE 1

Summary of follicle counts made on representative ovaries from each group of animals

	NUMBER OF OVARIES REPRESENTED	AVERAGE NUMBER OF STRUCTURES PER OVARY					TOTALS
		Primordial follicles	Primary follicles	Growing follicles	Vesicular follicles	Corpora lutea	
Normal.	5	38	10	27	21	31	127
Estrin injected.	5	48	20	39	8	0	115
Estrin and maturity factor.	5	42	12	31	9	49	143
Estrin and pituitary substance.	5	45	29	27	3	25	124
Estrin and APL.	5	46	38	12	3	26	125

The vaginal smears of the 60-day estrin-injected animals showed only cornified cells after the first three or four weeks of treatment. The uteri showed a proliferative type of endometrium, with changes simulating glandular cystic hyperplasia of the human endometrium. In many instances, severe intra-uterine infection was present; this resulted, apparently, from the prolonged stimulation with estrin. When gonadotropic hormones were injected after the long-term estrin injections, the smears changed, in most cases, to a mixture of leucocytes and nucleated epithelial cells. The endometria of these animals showed changes comparable to those characteristic of pseudopregnancy.

The ovaries of the animals which received large amounts of estrin after the long continued injection of a smaller amount showed no effect of the increased dosage. They were similar in weight (chart 1) and histologic appearance to the ovaries of the 60-day estrin-injected controls.

The pituitaries of all of the experimental animals were markedly increased in weight (chart 1). In general, the pituitaries of the animals

which received gonadotropic hormone or large doses of estrin showed a slight increase in weight over that observed in the 60-day estrin-injected controls. The implantation into immature mice of pituitaries from 60-day estrin-injected animals or from animals which had received both estrin and gonadotropic hormone produced no demonstrable change in the ovaries of the mice.

The adrenals of the 60-day estrin-injected animals showed no significant change in weight, when compared with those of the uninjected controls (chart 1). The adrenals of the animals which received, in addition, gonadotropic hormone or large doses of estrin were slightly hypertrophied.

The vaginal smears of the animals which were allowed access to a male following the 60-day period of estrin injections showed occasional recurrences of estrus but no return of regular estrous cycles. One of these animals became pregnant and delivered a normal litter four months after the injections were discontinued.

DISCUSSION. The results described above furnish strong evidence that the ovarian atrophy which results from long-term injections of estrin is due to a decrease in the amount of gonadotropic hormone available to the ovary. The fact that follicle growth, corpus luteum formation and arrest of follicular atresia can be produced in these atrophic ovaries by the injection of gonadotropic hormones indicates that the ovary is not fundamentally damaged by long continued estrin administration but is able to respond in an essentially normal manner if adequately stimulated. Since the gonadotropic hormones were given simultaneously with estrin, the results also indicate that estrin does not materially interfere with the action of gonadotropic substances upon the ovary.

The pituitaries of animals injected with estrin for a long period of time exhibited no ovarian stimulating capacity, either when implanted into immature mice or when subjected to the action of larger doses of estrin. This suggests that estrin acts directly on the pituitary, producing, after a sufficient length of time, a state of exhaustion with respect to its gonadotropic factor(s). Apparently other pituitary factors are not similarly affected. When large amounts of estrin were injected after the long continued administration of a smaller dosage, hypertrophy of the adrenals was noted. In view of our previously reported observation (5) that estrin administration produced no change in the size of the adrenals of hypophysectomized animals, this hypertrophy would seem to indicate the release of some adrenotropic factor by the pituitary. Moreover, in the present experiments, the effects of anterior-pituitary-like hormone upon the ovaries were qualitatively similar to those produced by gonadotropic preparations of the pituitary gland. This is not true with respect to the ovaries of hypophysectomized animals (14-15).

SUMMARY AND CONCLUSIONS

Long-term injections of estrin produced a decrease in ovarian size with a general regression of the germinal and interstitial elements. Ovaries which had become atrophic as a result of such treatment responded in a normal physiologic manner to injected gonadotropic hormones.

Long-term injections of estrin caused a marked pituitary hypertrophy. The adrenals were not significantly changed in weight by this treatment. The addition of gonadotropic hormones or of massive doses of estrin caused, in the majority of instances, further hypertrophy of the pituitary and a slight hypertrophy of the adrenals.

These results indicate that estrin acts directly on the pituitary, causing, after long periods of injection, an exhaustion of gonadotropic hormone production by the gland. The ovary is not fundamentally damaged by long continued estrin administration but becomes atrophic because of inadequate stimulation.

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THE BLOOD VOLUME OF NORMAL DOGS

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The evaluation of experimental procedures involving the circulatory, respiratory, and renal systems of dogs is dependent in a large measure upon an accurate quantitative determination of the changes in circulating blood volume occurring under such experimental conditions, and upon knowledge of the extent to which such changes constitute deviations from the normal state. As a control for a series of such experimental studies we have determined the plasma, cell and total blood volume in 50 normal dogs. The data so obtained have been analyzed to determine the normal range of blood volume, and to correlate the normal blood volume with body weight, the hemoglobin content of venous blood and the blood velocity rate.

Various authors have reported the total blood volume of normal dogs in terms of percentage of body weight. Thus, Arnold, Carrier, Smith and Whipple (1), using the carbon monoxide method, found the blood volume to average 7.78 per cent of body weight. McQuarrie and Davis (2), using the acacia method and the Pulfrich refractometer, obtained an average value of 9.76 per cent of body weight. Using various modifications of the original dye method of Keith, Rowntree and Geraghty (3), Smith, Arnold and Whipple (4) reported 10.39 per cent; Hooper, Smith, Belt, and Whipple (5) 10.1 per cent; Harris (6) 7.6 per cent; and Powers, Bowie and Howard (7) 11.91 per cent as the average percentage of body weight for normal total blood volume.

In terms of cubic centimeters per kilogram of body weight average values heretofore reported vary widely. From the data presented by McQuarrie and Davis (2) an average value of 97.6 cc. per kgm. is obtained. Arnold, Carrier, Smith and Whipple (1) found an average of 84.3 cc. per kgm., while the average value reported by Powers, Bowie and Howard (7) is 112.8 cc. per kgm. Further analysis of the findings of these three groups of workers shows a disagreement as to the relationship of unit volume in terms of body weight to variations in body weight. In the 19 dogs studied by McQuarrie and Davis (2) unit volume ranged from 73 cc. per kgm. to 114 cc. per kgm., and tended toward a constant value with increase in weight. In the series of 14 dogs reported by Smith, Arnold and Whipple

(4) unit volume varied from 89.9 cc. per kgm. to 125.3 cc. per kgm., and tended to increase with increasing weight. In the series of Powers, Bowie and Howard (7) unit volume varied from 96.0 cc. per kgm. to 130.0 cc. per kgm., and showed a marked decrease with increase in weight.

MATERIAL STUDIED. Dogs used in this study were mongrels, of widely varying breed mixtures. They were fasted for twelve hours without restriction of fluids before each volume determination. The dogs were loosely secured on a standard animal operating table during the determination, care being taken to prevent stasis in the limbs, and that the

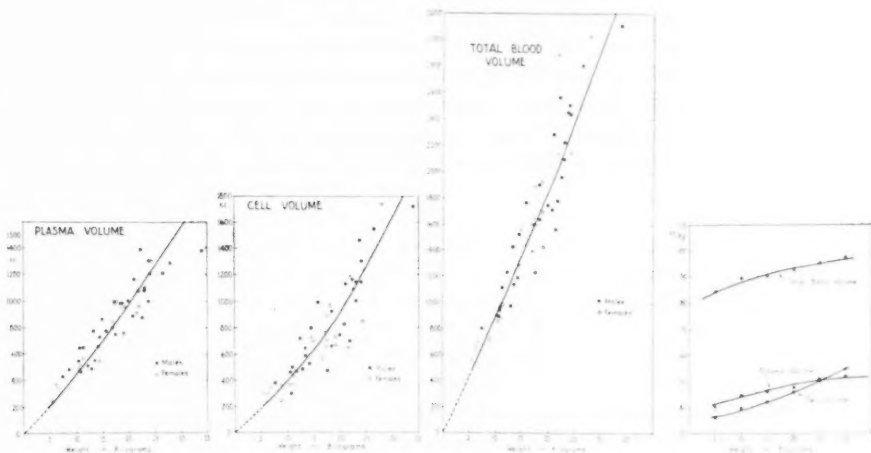


Fig. 1

Fig. 1

Fig. 1

Fig. 2

Fig. 1. Absolute plasma, cell and total blood volume in relation to body weight in 50 normal dogs.

Fig. 2. Unit plasma, cell and total blood volume in terms of cubic centimeter per kilogram of body weight in relation to body weight.

animal remained as quiet as possible throughout the procedure. No sedative, analgesic or anesthetic drugs were employed.

METHODS. Plasma, cell and total blood volumes were determined by the method developed by Gregersen, Gibson and Stead (8), as modified by Gibson and Evans (9). Hemoglobin values of venous blood were determined by the method of Evelyn (10). Blood velocity rates were determined by the cyanide method of Loevenhart, Schlomovitz and Seybold (11), from 0.2 cc. to 0.3 cc. of a 0.5 per cent solution of sodium cyanide being injected into a femoral vein. A stop-clock was used for timing the reaction constituting the end point.

RESULTS. Plasma, cell and total blood volumes, both absolute and in

terms of body weight, together with hemoglobin and hematocrit values and blood velocity rates as determined in 50 normal dogs are given in table 1. The range of weight of all animals was from 5.6 kgm. to 34.0 kgm. In figure 1 are shown the absolute plasma, cell and total blood volumes in relation to weight. Extremes in individual determinations ranged from 252 cc. to 1,385 cc. for plasma volume, from 245 cc. to 1,740 cc. for cell volume, and from 556 cc. to 3,100 cc. for total blood volume. Some degree of deviation of individual volumes from the curve of average values by weight groups occurs. In the case of total blood volume 72.0 per cent of the determinations fall within plus or minus 10 per cent of average value; whereas 66.1 per cent of the values for plasma volume and only 42.0 per cent of the values for cell volume fall within these limits. No appreciable difference in range of volumes exists between male and female dogs.

In figure 2 are shown the relationships of unit plasma, cell and total blood volume in terms of cubic centimeters per kilogram of body weight to variations in weight, the values plotted having been derived from the

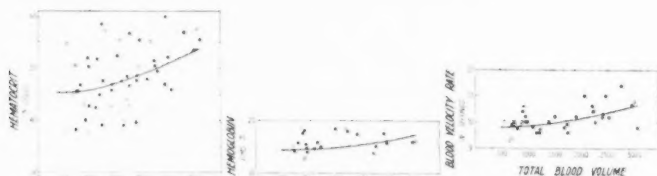


Fig. 3. Relationship of hematocrit and hemoglobin values of venous blood, and blood velocity rate to total blood volume.

average curves constructed in figure 1. It is apparent that unit volume is not a constant value but increases with increasing weight, and that with increasing weight the unit cell volume tends to increase more rapidly than the unit plasma volume.

The relationship of hematocrit and hemoglobin values and blood velocity rates to total blood volume are shown in figure 3. With increase in total blood volume there is an increase in all three values.

DISCUSSION. Comparison of results obtained in this study with those previously reported in the literature presents some difficulty, because of the differences in the methods and technique employed. In the entire series of dogs studied by Harris (6) volume determinations were made with the animals anesthetized. It is our experience that anesthesia is accompanied by a considerable reduction in circulating plasma volume and an increase in cell volume. These results can hardly be considered to represent true basal total blood volume.

Gregersen, Gibson and Stead (8) have shown the dye methods heretofore

employed to be subject to a number of sources of error. Among the more important of these are errors in colorimetry when the Duboseq or related types of comparison colorimeters are used for measurement of dye concentration in serum samples. Even diluted serum is more or less turbid, and in obtaining a color match between standard and unknown, the raising or lowering of the plunger in the cup containing the unknown results in differences in the amount of light dispersed by these turbid solutions on the two sides of the Duboseq instrument. As a result, unless the standard and unknown are of approximately equal dye concentration, large errors in estimation of the dye concentration of samples, and hence in the calculated volume will occur. Gregersen (12) obtained evidence that dilution of dyes with water or saline, or variations in the protein content resulting from dilution of dyed serum, has the effect of changing the absorption of such dye solutions both as to wavelength of maximum absorption and optical density. Therefore large errors may be introduced by dilution of serum samples to quantities sufficient for reading in the standard Duboseq colorimeter. Colorimetric errors of this type are minimized by the method we have employed (9).

Errors due to hemolysis, the presence of which in serum or plasma samples may be masked by red dyes are minimized by the use of a blue dye having a maximum absorption at $620\text{ }\mu\mu$, a wave-length at which the absorption of oxy-hemoglobin is minimal. By means of the spectrophotometer correction of errors due to the presence of oxy-hemoglobin in large amounts in either dye free or dyed samples is readily made (9).

In most of the techniques used by the authors referred to above, the volume calculation was based on the dye concentration of a sample taken three minutes after dye injection, under the assumption that dye mixing was complete in this time and that little or no dye had been removed from the circulation during the period of mixing. In our experience, mixing of dye, injected in 1 cc. or 2 cc. amounts, is never complete in three minutes, requiring on an average eight minutes. During this time considerable dye has been removed from the circulation. In the method we have employed, these sources of error are minimized by the procedure of basing the volume calculation on a dilution value obtained by the extrapolation of the slope of disappearance of the dye from the blood stream as determined from the dye concentration of from four to six serial blood serum samples taken at regularly spaced intervals from ten to thirty minutes after dye injection, to the time of dye injection. *The dilution value so determined represents the theoretical value that would obtain were all the dye to be mixed with all the blood before any dye has left the blood stream or any blood has been withdrawn in sampling, and represents the true plasma volume at the time of dye injection.*

Total blood volume expressed in terms of percentage of body weight

TABLE 1

Plasma, cell and total blood volume; hematocrit and hemoglobin values of venous blood, and blood velocity rate in 50 normal dogs

DOG NO.	SEX	DATE	WEIGHT	BLOOD VOLUME						HEMA-TOCRIT	HEMO-GLOBIN	BLOOD VELOCITY RATE
				Plasma		Cell		Total				
				kgm.	cc.	cc./kgm.	cc.	cc./kgm.	cc.	cc./kgm.	per cent	grams per cent
1	M	9/21/36	11	464	42.25	502	45.55	966	87.8	51.9	15.3	11
2	M	9/21/36	10.9	642	58.8	300	26.9	942	85.7	37.6	12.85	13
3	F	9/21/36	17	804	47.3	591	34.9	1,395	82.2	42.5		
4	F	9/28/36	10.5	490	46.6	372	35.5	862	82.1	43.1	13.5	9.5
5	M	9/28/36	8.8	480	54.5	245	33.7	725	88.2	38.1		9.5
6	M	10/ 6/36	14.3	655	45.8	533	37.2	1,188	83	44.8	15.7	8
7	M	10/ 6/36	13.6	550	40.4	590	43.3	1,140	83.7	51.66	14.5	
8	M	10/ 6/36	23.8	990	42.7	1,460	60.3	2,450	103	59.96	12.5	11.5
9	M	10/14/36	7.7	428	55.5	378	27.6	806	83.1	46.77	15.7	8.7
10	M	10/19/36	13	485	37.02	491	37.18	976	74.2	50.17	14.65	10
11	F	10/19/36	11.4	565	49.56	418	36.64	983	86.2	42.6	13.95	10
12	F	10/19/36	8.8	350	39.77	363	41.33	713	81.1	50.9	15	9.5
13	F	11/ 6/36	10.9	513	47.07	375	34.33	888	81.4	42.16	13.1	10
14	M	11/ 6/36	10.6	543	51.2	460	34.0	903	85.2	39.86	12.5	12
15	M	11/16/36	11.3	643	56.8	472	42.0	1,115	98.8	42.36		9
16	M	11/16/36	13.3	773	58.12	642	49.08	1,425	107.2	45.73		10
17	M	1/11/37	22.7	875	39.5	1,090	39.6	1,965	86.5	55.4	17.46	11
18	M	1/18/37	20.8	887	42.6	828	39.7	1,715	82.3	48.4	18.4	9
19	M	2/ 8/37	15.8	775	49	990	62.5	1,765	111.5	56.6		8
20	M	2/15/37	12.3	510	41.4	720	58.6	1,230	100.0	58.5		8
21	M	3/ 1/37	14.6	727	49.7	798	54.5	1,525	104.2	52.36	18.4	11
22	M	3/ 1/37	34	1,380	40.7	1,720	50.6	3,100	91.3	55.46		9
23	M	3/15/37	26.6	1,205	45.3	1,545	60	2,800	105.3	56.96		17
24	M	4/ 9/37	22.3	1,385	62.2	1,175	51.8	2,560	114	45.8		11
25	M	4/ 9/37	24.1	1,295	58.6	1,145	42.6	2,440	101.2	46.9		11
26	M	4/22/37	23.1	1,084	47.0	1,146	49.7	2,230	96.7	51.4		13
27	M	4/22/37	23.1	1,095	47.5	1,005	43.5	2,100	91	47.83		15
28	M	5/ 3/37	21.2	1,160	54.7	1,130	53	2,290	107.7	49.3	15.1	10
29	M	5/ 3/37	24.1	1,200	49.7	1,300	53.8	2,500	103.5	52	15.7	15
30	F	5/ 3/37	28	1,280	45.8	1,740	61.9	3,020	107.7	57.5	16.2	14
31	F	12/10/35	22	960	43.6	1,180	53.7	2,140	97.4	55.2		
32	F	2/27/36	17.5	990	56.5	710	40.6	1,600	91.4	38.1		
33	F	2/ 4/36	21.5	916	42.6	649	30.2	1,565	72.9	41.5		
34	M	1/29/37	20.0	1,000	50.0	745	37.3	1,745	87.3	42.7		
35	M	8/11/35	17.7	750	42.2	480	27.1	1,230	69.5	39.0		
36	M	7/14/35	21.8	1,077	49.3	703	32.2	1,780	81.7	39.5		
37	M	7/21/35	18.3	982	53.6	923	50.4	1,905	104.2	48.5		
38	M	8/ 4/35	18.4	980	53.2	660	35.8	1,640	89.3	38.8		
39	F	3/22/37	18.8	962	51.2	968	51.2	1,930	103	50.1		
40	F	3/23/37	19.1	756	39.6	669	35	1,425	74.6	46.8		
41	F	3/25/37	17.4	998	57.2	657	37.9	1,655	95.1	39.9		
42	F	4/13/37	17.2	842	48.9	761	44.4	1,603	93.3	47.5		

TABLE 1—*Concluded*

DOG. NO.	SEX	DATE	WEIGHT	BLOOD VOLUME						HEMA-TOCIT	HEMO-GLOBIN	BLOOD VELOCITY RATE
				Plasma		Cell		Total				
				cc.	cc./kgm.	cc.	cc./kgm.	cc.	cc./kgm.	per cent	grams per cent	seconds
			kgm.									
43	F	4/13/37	24.4	1300	53.3	850	34.9	2,150	88.2	42.1		
44	F	5/21/37	19.0	965	50.7	732	38.6	1,697	89.3	43.8		
45	F	5/21/37	14.5	550	38.2	734	50.4	1,284	88.6	57.2		
46	F	6/ 2/37	6.4	361	56.4	296	46.1	657	102.5	45.1		6.5
47	F	6/ 2/37	5.6	252	45.0	304	54.3	556	99.3	54.7		10
48	F	6/ 2/37	6.8	347	51.2	351	51.3	698	102.5	50.3		7
49	F	6/ 8/37	17.6	992	56.2	898	51	1,890	107.2	47.6		
50	F	6/ 8/37	15	862	57.3	698	46.7	1,560	104.0	55.3		

ranged from 8.4 per cent to 9.7 per cent, or in terms of unit volume from 84 cc. per kgm. in 5 kgm. dogs to 97 cc. per kgm. in 30 kgm. dogs. These values are lower than those obtained in two of the series referred to above (2), (7). The higher values of these authors are to be explained by their practice of basing plasma volume calculation upon the dye concentration of a single blood sample, which, as described above, always gives a falsely low dye dilution value, and hence falsely high plasma volume.

The finding of a definite increase in unit volume with increasing weight is in keeping with knowledge of the capacity of various tissues for holding blood. Thus, muscle is blood-rich, and fat blood-poor. The unit volume of fat dogs is less than that of muscular dogs, as illustrated by the following two protocols:

3/25/37. Dog X-1. Weight 38.2 kgm.

Plasma volume..... 1,420 cc.; 37.1 cc./kgm.
 Cell volume..... 980 cc.; 25.7 cc./kgm.
 Total blood volume..... 2,400 cc.; 62.8 cc./kgm.

4/22/37. Dog X-2. Weight 40.0 kgm.

Plasma volume..... 1,117 cc.; 27.9 cc./kgm.
 Cell volume..... 1,653 cc.; 41.4 cc./kgm.
 Total blood volume..... 2,770 cc.; 69.3 cc./kgm.

In both instances, values for unit volume fall well below the average value for the group of dogs shown in figure 1. It is our observation that the weight of the heavier dogs in this series was due largely to muscle, a blood-rich tissue. In other words, total blood volume bears a direct relationship chiefly to the amount of muscular tissue in the animal. A similar relationship of total blood volume to the proportion of blood-rich to blood-poor tissue has been found to exist in humans by Gibson and Evans (13).

In support of this view is the finding of slight but definite increases in

hematocrit and hemoglobin values both with increase in weight (table 1) and with increase in total blood volume (fig. 3). In as much as increased concentration of cells as evidenced by increasing hematocrit value results in increased viscosity of the blood it is entirely consistent that there should be a slight slowing of the blood velocity rate with increase in body weight and total blood volume (fig. 3).

CONCLUSIONS

1. Plasma, cell and total blood volume, hematocrit and hemoglobin value of venous blood, and blood velocity rate were determined in 50 normal mongrel dogs.

2. In terms of cubic centimeters per kilogram in dogs of from 5 to 30 kgm. in weight, plasma volume ranged from 41.2 cc. to 51.7 cc.; cell volume from 36.4 cc. to 54.6 cc.; and total blood volume from 84.0 cc. to 97.3 cc.

3. No distinct difference in plasma, cell or total blood volume in relation to weight exists between male and female dogs.

4. With increasing body weight, there is an increase in absolute plasma, cell and total blood volume, and an increase in unit plasma, cell and total blood volume expressed in cubic centimeters per kilogram of body weight. Unit cell volume tends to increase more rapidly than unit plasma volume.

5. With increase in body weight, and the accompanying increase in total blood volume, there is an increase in hematocrit and hemoglobin values, and a slowing of blood velocity rate.

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